New combination and new synonymy in *Piptadenia* (Fabaceae: Mimosoideae)

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ABSTRACT

Mimosa retusa Jacq. is found to be identical in several features to the species currently known as *Piptadenia flava* (Spreng. ex DC.) Benth. The transfer of *Mimosa retusa* to *Piptadenia* is based on similarities in leaflet venation determined by comparison to authentic material of *Piptadenia flava.* Importantly, neither the venation nor petiolar glands are identical to those of *Senegalia riparia* (Kunth) Britton & Rose, which was previously considered a synonym of *Mimosa retusa* by Howard. Based on morphological similarities, *Acacia plumosa* Mart. ex Colla, is now recognized to be synonymous with *Piptadenia trisperma* (Vell.) Benth. Also, the name *Mimosa trisperma* Vell. is herein lectotypified. Published on-line www.phytologia.org *Phytologia 102(1): 1-4 (March 22, 2020).* ISSN 030319430.

KEY WORDS: *Acacia* s. l., Fabaceae, Mimosoideae, new combination, new synonymy, *Piptadenia flava*, *Piptadenia retusa*, *Piptadenia trisperma*, *Senegalia riparia*.

Many difficulties remain for placement of mimosoid legume taxa in appropriate genera and assignment of names that are in accord with current concepts of nomenclature (Turland et al., 2018). The present examination represents a contribution toward a monographic treatment of the mimosoid legumes of the New World.

Piptadenia retusa (Jacq.) P. G. Ribeiro, Seigler & Ebinger, comb. nov.

A taxon originally described by Jacquin (1760) as *Mimosa retusa* is transferred to *Piptadenia* Benth. The affiliation is based primarily on distinctive features of the taxa: leaflet venation and the structure of the petiolar gland. Those of the type specimen [*N. J. Jacquin s. n.*, Colombia: "[F]rom Cartagena", BM] were compared to authentic material of *Piptadenia flava* (Spreng. ex DC.) Benth. Because the type consists of only a portion of a leaf, there is a paucity of data available for study.

Leaflet venation of both *Piptadenia retusa* (Figure 1) and *P. flava* (Figure 2) is raised on the abaxial surface and consists of a midvein and two or three veins arising from the base of the leaflet and are sloped toward the apex of the leaflets. Typically, 3 to 5 other veins arise from the midvein and slope toward the leaflet apex. In contrast, the venation of *Senegalia riparia* (Kunth) Britton & Rose in Britton & Killip (Figure 3) is not raised except for a subcentral midvein and a weaker vein from the base. Most other venation is indistinct, but often oriented perpendicularly to the midvein.

The petiolar gland of *Piptadenia retusa* and *Piptadenia flava* is normally solitary (Ribeiro, 2017, mentions a rare occurrence of two glands), near the middle of the petiole, fused to and raised above the

petiolar groove, oblong to elliptic, 2.5--5.1 mm long, apex depressed to cup-shaped and glabrous. The petiolar glands of *Senegalia riparia*, in contrast, are usually 2, one located near the middle of the petiole, generally larger than the second, which is just below the lowermost pinna pair, sessile to subsessile, mostly oval to orbicular, 0.5--2.2 (4.0) mm across, commonly flattened to shallowly cup-shaped, glabrous.

Because the name of Jacquin (1760) is older than that of Velloso [1827 (1831)], the name for this taxon must be *Piptadenia retusa* (Jacq.) P. G. Ribeiro, Seigler & Ebinger.

Piptadenia retusa (Jacq.) P. G. Ribeiro, Seigler & Ebinger, **comb nov.** Basionym: *Mimosa retusa* Jacq., Enum. Syst. Pl., 34. 1760. *Acacia retusa* (Jacq.) R. A. Howard, J. Arnold Arbor. 54(4): 459. 1973. – **TYPE**: Colombia: "[F]rom Cartagena," *J. N. Jacquin, s.n.* (holotype, BM). [= *Acacia flava* Spreng. ex DC., Prodr. (A. P. de Candolle) 2: 469. (Nov.) 1825; *Piptadenia flava* (Spreng. ex DC.) Benth., Trans. Linn. Soc. London 30(3): 371. 1875; *Piptadenia communis* var. *stipulacea* Benth., Fl. Brasil. (Martius) 15: 279-280. 1876; *Piptadenia leptocarpa* Rose, Contr. U.S. Natl. Herb. 1(9): 325-326. 1895; *Mimosa buceragenia* B. L. Rob., Proc. Amer. Acad. Arts 43(2): 23. 1908 [1907]; *Piptadenia stipulacea* (Benth.) Ducke, Arch. Jard. Bot. Río de Janeiro 5: 126. 1930; *Pityrocarpa flava* (Spreng. ex DC.) Brenan, Kew Bull. 10(2): 176. 1955; *Pityrocarpa stipulacea* (Benth.) Brenan, Kew Bull. 10(2): 177. 1955; *Mimosa carbonalis* A. Molina, Ceiba 18(1-2): 102-104 1974].

The imperfectly known name *Mimosa carthagenensis* Mill. based on Houstoun collections and a later plate "Carthagena in New Spain," plate CCXCI (291) in Miller (1760: 194) was referred to *Acacia retusa* by Rudd (1976). Based on her judgement, the name *Mimosa carthagenensis* Mill. may possibly be referred to *Piptadenia retusa.*

Although a fruit was reported to be associated with the type specimen of *Mimosa retusa* and is mentioned in a later description (Jacquin, 1763), only a leaf fragment was mentioned in the original description (Jacquin, 1760; Rudd, 1976).

Piptadenia trisperma (Vell.) Benth.

In 1827, Martius sent a series of specimens to Colla in Torino, apparently for identification. Among those was a specimen bearing the name *Acacia plumosa* in Martius' handwriting. On the reverse side of this label, the description that appears in Colla (1834) is written in Colla's handwriting (Seigler et al., 2013). The specimen at TO has spicate inflorescences and prickles, some of which are paired at the nodes, but are also scattered along the petioles. The petiolar gland of *Piptadenia trisperma* is solitary, near the middle of the petiole, sessile, oblong, 1.8--2.5 (4.0) mm across, apex flattened to depressed, glabrous. Leaflet venation is more or less central. These characters are quite similar to those of *Piptadenia trisperma* (Vell.) Benth. (Ribeiro, 2017), which is the most likely taxonomic placement for Martius' *Acacia plumosa.*

Piptadenia trisperma (Vell.) Benth., J. Bot. (Hooker) 4(31): 337. [Dec.] 1841. Basionym: *Mimosa trisperma* Vell., Fl. Flumin. Icon. 11: tab. 40. 1827 [29 Oct. 1831], Fl. Flumin., 440. 1881 [Latin descr.] **TYPE**: Brazil. (lectotype, designated here, Fl. Flumin. Icon. 11: tab. 40. 1827 [29 Oct. 1831], Fl. Flumin., 440. 1881 [Latin descr.]; *Acacia trisperma* (Vell.) Mart., Flora. 20(2), Beiblätter 8: 108--109. [Herb. flor. bras.] 1837. *Pityrocarpa trisperma* (Vell.) Brenan, Kew Bull. 10(2): 177. 1955. [= *Acacia plumosa* Mart. ex Colla, (Jul.) 1834.]

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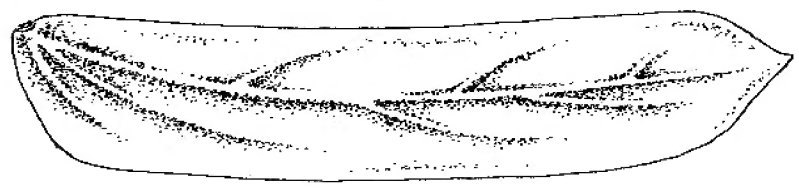

Fig. 1. *Piptadenia retusa* (Jacq.) P. G. Ribeiro, Seigler & Ebinger. A. Abaxial surface of leaflet. *N. J. Jacquin s. n.* Colombia, Cartagena (BM).

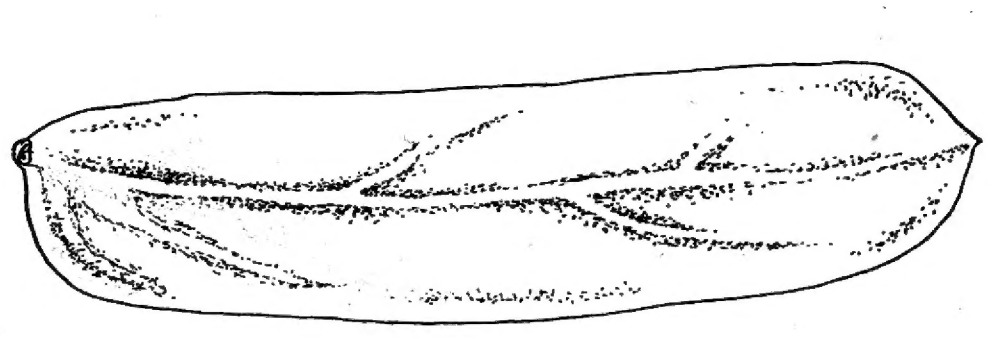

Fig. 2. *Piptadenia flava* (Spreng ex DC.) Benth. Abaxial surface of leaflet. *J. A. Steyermark, J. Hoyos, & B. Holst 130986.* Venezuela. Nueva Esparta. Isla de Margarita. Cerro El Maco, via Santa Ana, 23 Mar 1985 (MO).

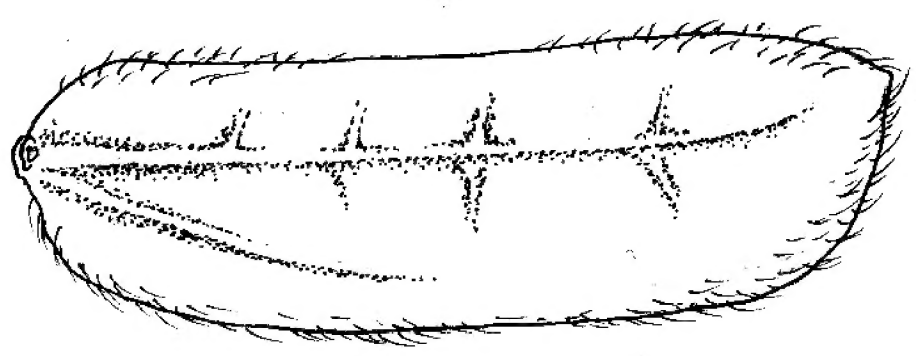

Fig. 3. *Senegalia riparia* (Kunth) Britton & Rose ex Britton & Killip. Abaxial surface of leaflet. *A. Gentry, H. Cuadros & P. Keating 60597.* Colombia, Bolivar. Santuario Nacional de los Colorado, Municipio San Juan Nepomuceno, 70 km SW of Cartagena. 9º 58' N 75º 10' W, 11 Jan 1988 (MO).

A nomenclatural and systematic note on the genus *Myiophagus* (*ex* Chytridiomycota)

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ABSTRACT

We reaffirm the opinion that the spelling of this fungal genus-name should be *Myiophagus*, not *Myrophagus*, and that correct authorship is *Myiophagus* Thaxter ex Sparrow, or simply *Myiophagus* Sparrow. Although two species have been published under *Myiophagus—M. ucrainicus* (M.C. Wize) Sparrow (upon which the genus was originally based), and *M. characeus* Kiran & Dayal—the propriety of inclusion of the latter species is uncertain (although it is here tentatively retained in the genus). Morphologically, placement of *Myiophagus* (based on *M. ucrainicus*) in the Blastocladiomycota seems most supportable, among several suggestions made for its classification. It is not clear, though, with which family (of Blastocladiales) *Myiophagus* has relationship. In any case, molecular data are essential to ultimate systematic decisions regarding the genus; interested investigators are encouraged to pursue study of *Myiophagus*—whenever it may be found again, collected and cultured. Published on-line www.phytologia.org *Phytologia 102(1): 5-8 (March 22, 2020).* ISSN 030319430.

KEY WORDS: Dipteran, larvae, nuclear-cap, orthography, pupae, resting-spores, thallus, zoospores.

Sparrow (1939) described a new genus of Fungi, with posteriorly uniflagellate zoospores, which he considered a Chytridiomycete; this organism occurred on certain dipteran pupae. Material examined by Sparrow had been collected by Dr. Roland Thaxter (in 1902) who made unpublished, descriptive notes on the specimens; this material (and information) was shown to Sparrow, in 1927, Thaxter encouraging Sparrow to pursue the project. Sparrow, unable to find additional specimens in the field, studied Thaxter's herbarium material, descriptive information, and drawings. Sparrow (1939) then validly published genus "*Myrophagus*"—purportedly using Thaxter's proposed spelling—generously (but mistakenly) crediting Thaxter (alone) with the *published* genus.

Sparrow (1939) noted that a similar organism (from generally similar substrates: larvae of kinds of beetles/weevils, in this case) had been described by M.C.Wize (1904) from the Ukraine (actually, the first published description of the organism, if only its resting spores, later to be called *Myiophagus*; Wize had referred it to genus *Olpidiopsis*). Regardless of size differences, Sparrow concluded the specimens seen by Wize and those seen by Thaxter represented the same taxon. Sparrow accordingly transferred Wize's species, "*Olpidiopsis urcrainica*," to "*Myrophagus*" as a new combination, *M. ucrainicus* (Wize) Sparrow (1939).

Torrey (1945) noted that Thaxter (in 1915) had shown him material of the "fly-inhabiting chytrid," which Torrey (1945) realized was the organism described by Sparrow (1939). Torrey indicated that he (Torrey) *correctly* copied, from Thaxter's 'script,' the name *Myiophagus*, not *Myrophagus*, the original notation (by Thaxter) seemingly not later preserved (cf. Torrey, 1945, p. 161, 2nd paragraph under '*Myiophagus*'). After studying the orthography of the name, Torrey concluded that Sparrow's (1939) spelling was in error, and effected change of the generic name to *Myiophagus*—not affecting Sparrow's (1939) authorship credit for the genus (cf. Art. 33.1, 33.2, 60.1; ICNAFP). One could argue (Art. 60.1) that the original spelling by Sparrow ('*Myrophagus*')—though less appropriate in meaning—could be retained, if not for the fact that Sparrow (1960) later employed (thereby *de facto* accepting) Torrey's orthographic correction to '*Myiophagus*.' Pursuant to Torrey's spelling change, virtually all authors eventually accepted '*Myiophagus*' (rather than '*Myrophagus*') as the correct spelling, including Karling

(1948, 1977), Fisher (1950), Muma and Clancy (1961), Dick (2001), James et al. (2014), and IF (*Index Fungorum*, current).

Authorship of *Myiophagus* has remained confusing, though. Sparrow (1939) incorrectly cited the genus in his validating publication as '*Myrophagus* Thaxter' (Thaxter proposed, but did *not* publish, the genus). Pursuant to this, Fisher (1950) cited a 'find' of this organism as '*Myiophagus* sp. Thaxter.' Karling (1977) and Dick (2001) noted authorship of *Myiophagus* as 'Thaxter in Sparrow'—incorrect, since Thaxter did not publish *in* Sparrrow (1939). Humber (2012) continued to list authorship as '*Myiophagus* Thaxter.' *Index Fungorum* (IF) indicates authorship as '*Myiophagus* Thaxt. ex Sparrow'—essentially a correct citation, since Thaxter suggested the genus but it was Sparrow (1939) who validated it in publication (though introducing a misspelling). The name (authorship) should be cited '*Myiophagus* Thaxter ex Sparrow,' or simply '*Myiophagus* Sparrow'—Sparrow being the sole publishing author (cf. Art. 46.7, ICNAFP).

Other than noting (Sparrow, 1939) that *Myiophagus ucrainicus* (zoospores posteriorly uniflagellate) could not be accepted in *Olpidiopsis* (zoospores laterally biflagellate)—where it was initially placed (M.C.Wize, 1904)—Sparrow was justifiably tentative about relationships of this "chytrid." Sparrow mentioned possible connections to *Olpidium* and (perhaps oddly to) *Woronina*, or with *Micromyces* and *Synchytrium*; but, none of these suggestions were made with assurance. Though using a question-mark, Sparrow (1942) parenthetically listed "*Myrophagus*" under Olpidiaceae (Chytridiales). Karling (1948) explored the idea of relationship of *Myiophagus* within the Achlyogetonaceae (Chytridiales), particularly with *Septolpidium* (although thalli of *Septolpidium* do not form isthmuses; see below) or, perhaps less enthusiastically, with the Blastocladiales. Karling (1977) became more open to the idea of relationship with Blastocladiales, but nonetheless retained *Myiophagus* provisionally in the Achlyogetonaceae (Chytridiales). Relationships of *Myiophagus* remained uncertain; it was relegated (Dick, 2001) to a group of 'Miscellaneous Genera' of undetermined position. *Myiophagus* is placed in the Chytridiales in IF, no further relationship indicated. Uncertainty about morphological comparisons of *Myiophagus* (to potentially similar fungi) thus seems to persist. Whereas knowledge of life-cycle stages of *M. ucrainicus* has been pieced together by several investigators—the stages seemingly established—understanding of developmental biology of this organism could benefit from further study.

Although relationships of *Myiophagus* are unresolved, in illustrations by Karling (1948, 1977) of *M. ucrainicus* similarity to Blastocladiales is evident. The presence in zoospores of *Myiophagus* of a grouping of apically positioned, small, non-refractive globules—or in some cases (Karling, 1977, p. 61, fig. 2) of an apparent nuclear-cap (above a rounded-triangular nucleus)—is suggestive of Blastocladiomycota. Zoospore-ultrastructure in this phylum is characteristic (James et al., 2014; Powell, 2016), featuring a nuclear-cap of ribosomes, a generally triangular nucleus, and a 'side-body [organellar] complex.' The mitotic figure in Karling (1977, fig. 14, p. 61)—showing intranuclear mitosis with a totally enclosed nuclear envelope—also suggests Blastocladiomycota (in contrast to Chytridiomycota where, in intranuclear mitosis, the nuclear envelope is open at the spindle poles, cf. Powell, 2016, p. 19). The thallus of *Myiophagus* (becoming catenulate, isthmuses connecting segments) and the 'roughened-reticulate' appearance of resting-spores (yellow- red- or orange-tinted, in powdery mass) are consistent with some members of this phylum. The classification of *Myiophagus* in Blastocladiomycota (Humber, 2012; James et al., 2014; Powell, 2016) thus appears correct. When young, the coenocytic thallus can resemble that of family Coelomomycetaceae. When older, the septate, catenated thallus is suggestive (save lack of rhizoids or rhizoid-like extensions) of thalli of the Catenariaceae. Molecular data would surely clarify potential relationships.

Myiophagus ucrainicus was initially considered rare, known just from material collected by Thaxter in 1902, and M. C. Wize (1904). However, additional collections mentioned—see, K. F. Wize (1929), Petch (1939, 1940), Waterson (1946), Fisher et al. (1949), Fisher (1950), Muma and Clancy

(1961), Karling (1948, 1977), Czeczuga and Godlewska (2001), and Czeczuga et al. (2003)—suggest broad distribution, though an organism probably no more than locally common. Karling (1948) discussed infestations ("chytridiosis") of scale-insects, on citrus, in Florida by *Myiophagus*; possible application in biological control of scale-insects has been mentioned (e.g., Karling, 1948; Fisher, 1950; Powell, 2016). Humber (2012, p.158) noted occurrence of *Myiophagus* on "terrestrial insects," indicating it is "rarely collected"—hence, 'rarity' may in part represent scant collecting. *Myiophagus* primarily infects insects (immature stages of Coleopterans, Dipterans and Homopterans). A report (not illustrated) from leeches in Poland (Czeczuga et al., 2003) suggests a wider range of invertebrate hosts; if confirmed as *Myiophagus,* one might wonder if this *possibly* represents an undescribed species.

Another species of *Myiophagus* has, in fact, been described—*M. characeus* Kiran & Dayal (1997), from India (found in the alga, *Chara*)—listed in IF, but not in Dick (2001). Descriptive information in Kiran and Dayal (1997) is relatively sparse; e.g., 'planospores' (zoospores) of '*M. characeus*' (stated to be posteriorly uniflagellate) were not individually illustrated (no internal detail given), with little basis for comparison to zoospores of *M. ucrainicus*. Some sporangia of '*characeus*,' unlike '*ucrainicus*,' possess elongate discharge-tubes. The tubular, syncytial thallus (of '*characeus*') becomes septate—this obscurely illustrated except for resultant(?), '*Olpidium*-like' segments—but no catenation is evident. The smooth, rather thin-walled (sac-like) resting-spores of '*characeus*' are quite unlike the thick, double-walled, reticulate resting-spores of '*ucrainicus*.' The status of *M. characeus*, as a species of *Myiophagus*, would have to be regarded as somewhat questionable; there is, however, an apparent resemblance of this organism to *Septolpidium* (Achlygetonaceae, Chytridiomycota; Kiran and Dayal, in fact, appear to suggest relationship of *M. characeus* to Achlyogetonaceae). Molecular sequence data could eventually resolve systematic placements of *M. ucrainicus* and *M. characeus*, and their possible relationship (if any) to one another. For now, we do not remove *M. characeus* from *Myiophagus*, in spite of rather striking differences from *M. ucrainicus*.

Taxonomic Summary of Genus *Myiophagus* (Blastocladiomycota): See full refs. in Lit. Cited.

Myiophagus R. Thaxter ex F. K. Sparrow (1939)—misspelled by Sparrow, *Myrophagus*, an orthographic error corrected to *Myiophagus* (Torrey, 1945; presently the accepted spelling). Herbarium collection = R. Thaxter #994, Kittery Point, Maine, 18 Sept., 1902—specimens at Farlow Herbarium, Harvard, and The University of Michigan Herbarium (MICH 334103). Full-plate **illustrations** of *Myiophagus* are **available** in Karling (1948, p. 248; and 1977, p. 61) and are not here reproduced.

M. ucrainicus (M.C. Wize) F.K. Sparrow (1939). Resting-spore photographs: Humber (2012, p.160).
Olpidiopsis ucrainica M. C. Wize (1904), original species; Ruthenian area, western Ukraine.
Entomophthora reticulata T. Petch (1939), Ingleborough, North Yorkshire, England.

M. characeus U. Kiran & R. Dayal (1997), Varanasi, India. Status uncertain (discussed above).

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Hybridization and introgression between *Juniperus communis* var. *saxatilis* and var. *hemispherica* in the Pyrenees Mountains, France.

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ABSTRACT

An investigation of variation between *J. communis* var. *hemispherica* and var. *saxatilis* in the Pyrenees revealed 5 SNPs and 1 indel (in nrDNA) that distinguished the varieties and revealed one var. *hemispherica* plant (15401) and 23 var. *saxatilis* plants. Plant 15401 had 3/5 informative sites typical of var. *hemispherica* and the other 2 bases were heterozygous, indicative of a backcross of var. *saxatilis* into var. hemispherica. Chloroplast (cp) petN-psbM did not clearly separate var. *hemispherica* and var. *saxatilis*. Although no plants of *J. c.* var. *hemispherica* was found, the presence of a backcross, indicates *J. c.* var. *hemispherica* either grows in the region or perhaps, long distance transfer of pollen. Published on-line www.phytologia.org *Phytologia 102(1): 9-13 (March 22, 2020).* ISSN 030319430.
KEY WORDS: *Juniperus communis* var. *saxatilis, J. c.* var. *hemispherica,* Pyrenees, hybridization, introgression, nrDNA, cpDNA, petN-psbM.

Juniperus communis L. is the only juniper species that grows in both the eastern and western hemispheres (Adams, 2014). It contains at least 10 varieties (Fig. 1) that are poorly resolved by nrDNA and cpDNA (Adams and Schwarzbach, 2013, Adams 2014). *Juniperus communis* var. *hemispherica* (J. & C. Presl.) Parl., described from a shrub growing on the flanks of Mt. Etna, Sicily, differs by 5 nrDNA SNPs and 1 indel (Adams and Schwarzbach, 2013) and seems one of the most distinct varieties (in its DNA, Fig. 1). Yet, the discovery of additional populations in Europe has been futile (Adams 2014). Prostrate juniper plants in Sierra Nevada, Spain, generally called var. *hemispherica*, not small shrubs as in Sicily. However, they do appear to be var. *hemispherica* (orange, Fig. 1).

To investigate the more northern range of *J. c.* var. *hemispherica*, we collected samples of horizontal, putative var. *hemispherica* from Pyrenees and examined nrDNA (ITS) and cp petN-psbM DNA sequences.

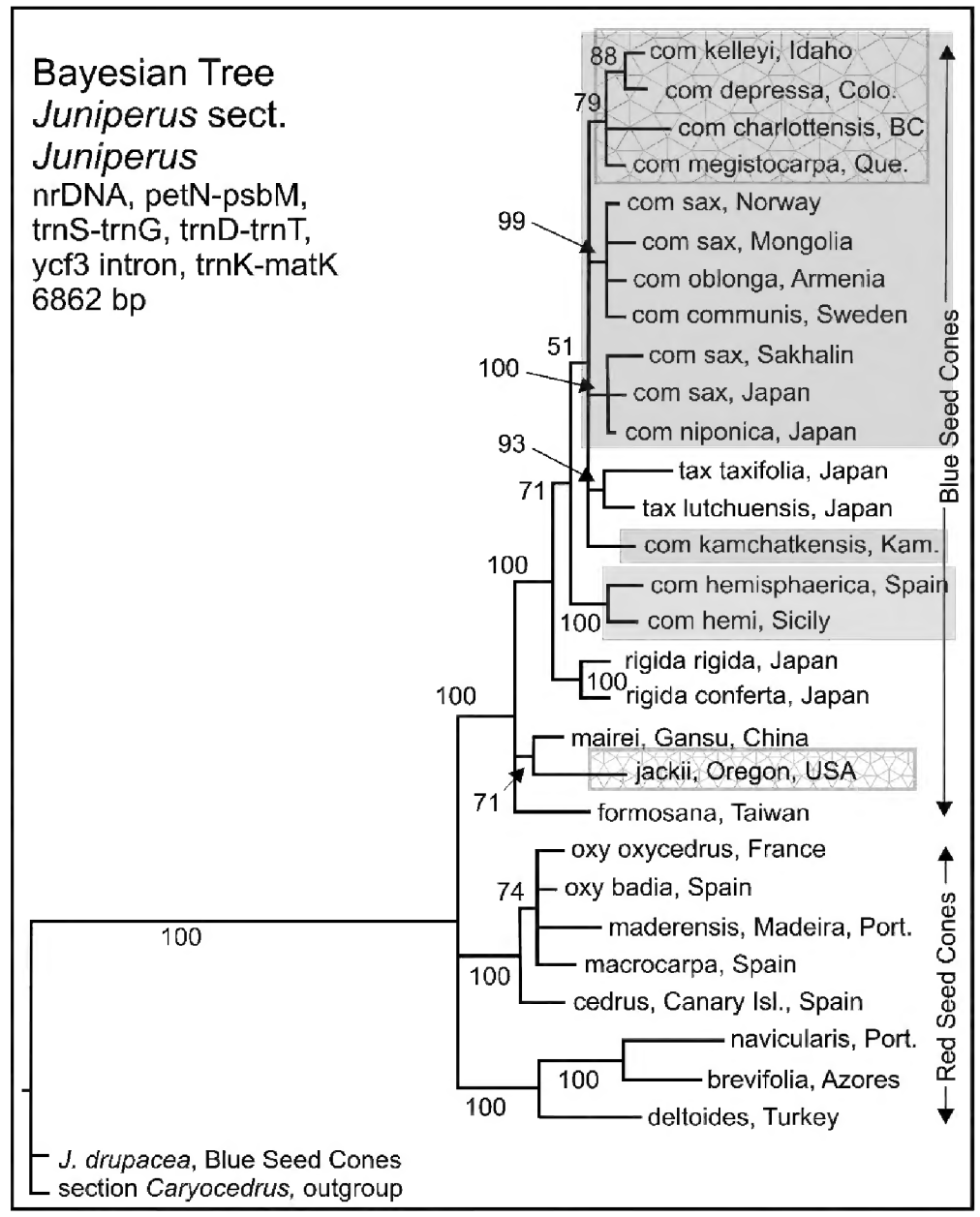

Figure 1. Bayesian tree of *Juniperus* sect. *Juniperus* (adapted from Adams and Schwarzbach, 2012).

MATERIAL AND METHODS

Specimens used in this study:
Juniperus communis var. *hemispherica*:
France:
horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42.907° N 02.943° E, 600 m, Feb. 2018, coll. *Marc Espeut, ns 1,2,3,4*, 23 Lab Acc. *Robert P. Adams 15401,15402,15403,15404,*
horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42° 54' 42.82" N, 2° 50' 37.47" W. 630 m, 8 March 2019, Coll. Marc Espeut ns 1-20, Lab Acc. Robert P. Adams 15581-15600(20), all horizontal, except 15598 was a sub-shrub.
Spain:
prostrate to 0.5m tall, with *J. sabina*. Sierra Nevada, s. of Granada, Spain. 37° 06' 17" N 3° 24' 51" W, 2100m, 20 Oct. 1993.Coll. *Robert P. Adams 7194-7195.*
prostrate to 0.2m tall x 3-5 m wide, abundant at Ski area, Sierra Nevada, s. of Granada, Spain. 37° 06' 02.54" N, 03° 24' 00.55" W 2024 m, 6 June 2019, Coll. *Robert P. Adams 15702-15703*

See Adams and Schwarzbach. (2013) for previously analyzed specimen locations.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS primers utilized. The primers for trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. 2.31 (Technelysium Pty Ltd.).

RESULTS

Analyses of 24 putative var. *hemispherica* plants revealed one var. *hemispherica* (15401) plant and 23 var. *saxatilis* plants (Table 1). Plant 15401 had 3/5 nucleotides typical of var. *hemispherica* and 2 sites that were heterozygous (purple, Table 1), indicative of its being a backcross of var. *saxatilis* into var. *hemispherica*. Data for *J. communis* var. *communis* (Sweden) and *J. c.* var. *saxatilis* (Norway) were included the analyses and it is interesting that they are not distinguished from *J. c.* var. *saxatilis* of Pyrenees (Table 1). The cp petN-psbM data did not clearly separate var. *hemispherica* and var. *saxatilis* (Table 1). Analyses of trnS-trnG, trnD-trnT, and trnL-trnF failed to find SNPs that distinguish var. *hemispherica* and var. *saxatilis* (Adams unpublished).

Two of the samples of var. *hemispherica* from Sierra Nevada (7194, 7195) have an A at site 1149 suggesting they are backcrossed from var. *saxatilis* (Table 1). They also have a T at cp petN-psbM site 305, that is common in the var. *saxatilis* from the Pyrenees. Two plants (15589, 15590, Table 1) are heterozygous at site 305 (W = A/T), but perhaps with is just a local mutation.

Table 1. SNPs and indels from nrDNA (ITS) and cp petN-psbM for *J. communis* from the Pyrenees. The 7 bp duplication-insert was not found in *Juniperus* sect. *Juniperus*, and appears to be unique to the Pyrenees. nrDNA sites highlighted in yellow are informative sites between var. *hemispherica* and var. *saxatilis* (and var. *communis*). Site values highlighted in Gold are unusual mutations, but not informative to distinguish known taxa.

		nrDNA (ITS) sites[1]										cp DNA (petN-psbM) sites**	
acc. no.	nrDNA classif.	Indel[2]	S1	S2	S3[2]	S4[2]	S5[2]	S6	S7	S8[2]	S9[2]	cp S1[2]	7 bp cp Indel
		205	277	305	400	404	414	428	602	642	1149	305	535
9045	hemi Sicily	TTT	C	T	A	C	C	C	A	T	G	C	GTAATTAC - - - - - - -
9046	hemi Sicily	TTT	C	T	A	C	C	C	A	T	G	C	GTAATTAC - - - - - - -
15702	hemi S Nev	TTT	C	T	A	C	C	C	A	T	G	C	GTAATTAC - - - - - - -
15703	hemi S Nev	TTT	C	T	A	C	C	C	A	T	G	C	GTAATTAC - - - - - - -
7194	hemi BC,t1 Sax S Nev	TTT	G	T	A	C	C	C	A	T	A	T	GTAATTAC - - - - - - -
7195	hemi BC,t1 Sax S Nev	TTT	G	T	A	C	C	C	A	T	A	T	GTAATTAC - - - - - - -
15401	hemi BC,t2 sax Pyr	TTT	C	T	A	C	C	C	A	Y	R	C	GTAATTAC - - - - - - -
11206	sax,Nor.	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
11207	sax,Nor.	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
7846	com,Swed	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
7847	com,Swed	---	C	T	G	T	T	G	A	C	A	C	GTAATTAC - - - - - - -
15583	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15586	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15587	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15589	sax Pyr	---	C	W	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15595	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15598	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15592	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTAC - - - - - - -
15581	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15582	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15584	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15585	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15588	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15403	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15591	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15594	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15596	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15597	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15599	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15600	sax Pyr	---	C	T	G	T	T	C	A	C	A	T	GTAATTAC - - - - - - -
15402	sax Pyr	---	C	T	G	T	T	C	G	C	A	C	GTAATTACTAATTAC
15404	sax Pyr	---	C	T	G	T	T	C	G	C	A	C	GTAATTACTAATTAC
15590	sax Pyr	---	C	W	G	T	T	C	A	C	A	C	GTAATTACTAATTAC
15593	sax Pyr	---	C	T	G	T	T	C	A	C	A	C	GTAATTACTAATTAC

[1]Indel 205: xxxTGCTGGACGG; S1,277:xGTGGATTCCC; S2,305:xCGGGCGCAAA; S3,400: GGACGTCCGx; S4,404: GGACGTCCGNGGCCx;S5,414: xTGAGATTT; S6,428: xTCGGTCGTG; S7,602: xCGACTCTCCC; S8,642: XGGGGCGGGG; S9,1149: xTCTTTGGTG.

**cp S1,305: xGAACCATAC; site 536:GTAATTACxxxxxxx, where xxxxxxx = TAATTAC or TATTACT (7bp insert).

[2]informative sites

The 7 bp duplication-insert at site 535 in cp petN-psbM (15402, 15404, 14409, 15593, Table 1) is not common in these Pyrenees data. The duplication-insert was not found in any juniper species in a search of *Juniperus* sect. *Juniperus*, (Adams and Schwarzbach, 2013) and appears to be unique to the Pyrenees population.

The discovery and verification of the presence of a backcross of var. *saxatilis* into var. *hemispherica* in the Pyrenees expands our knowledge of the range of *J. communis* var. *hemispherica*. Although it appears to be a small semi-globose shrub (hence the name *hemispherica*) near the type locality in Sicily (Fig. 2), it also grows as a nearly prostrate shrub on Mt. Etna, Sicily (Fig. 3). In the Sierra Nevada it is a very prostrate shrub (Fig. 4) and a nearly prostrate shrub in the Pyrenees, France (Fig. 5). Additional population research is needed to better understand its distribution.

Figure 2. *J. c.* var. *hemispherica* as a shrub at type locality, slopes of Mt. Etna, Sicily.

Fig. 3. *J. c.* var. *hemispherica*, spreading shrub, at a second location on the slopes of Mt. Etna. Photo from Pietro Miniscale.

Fig. 4. *J. c.* var. *hemispherica* as a very prostrate shrub, Sierra Nevada, Spain.

Fig. 5. *J. c.* var. *hemispherica* is a nearly prostrate shrub in the Pyrenees, France.

ACKNOWLEDGEMENTS

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Allopatric hybridization and introgression between *Juniperus scopulorum* Sarg. and *Juniperus blancoi* Martínez in northern Mexico: Unidirectional gene flow

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ABSTRACT

Analyses of nrDNA (ITS) and petN-psbM (cpDNA) confirmed that allopatric hybridization and introgression are occurring between *J. scopulorum* and *J. blancoi* in northern Mexico. Hybrids (by ITS data) all have *J. blancoi* var. *mucronata* as the maternal parent and *J. scopulorum* as the paternal (pollen donor) parent in Sonora and Chihuahua, with two backcrossed plants in the Guadalupe Mtns., TX and two hybrids discovered in northeastern Arizona. No hybrids or introgressants were found that contained the *J. blancoi* type chloroplast, suggesting the gene flow is unidirectional, north to south, from *J. scopulorum* to *J. blancoi*. Prevailing winds (Jan, Feb, March) give support to northern pollen flow hypothesis. Isolated occurrences of hybrids in northern Arizona seem more likely to be products of Pleistocene sympatry, and not modern northern pollen flow from *J. blancoi* in Mexico to northern Arizona because no *J. blancoi* chloroplasts (i.e., ex pollen) were found in the USA. It appears that *J. b.* var. *mucronata* is in the midst of a chloroplast capture event. Published on-line www.phytologia.org *Phytologia 102(1): 14-26 (March 22, 2020).* ISSN 030319430.

KEY WORDS: *Juniperus blancoi, J. scopulorum, nrDNA, petN-psbM,* hybridization, introgression, Pleistocene sympatry.

Variation in *Juniperus scopulorum* Sarg. has been reported using terpenoids (Adams 1983, 2011a). Recently, an extensive DNA analysis confirmed allopatric hybridization and introgression between *Juniperus maritima* R.P. Adams and *J. scopulorum* (Adams et al. 2010; Adams 2015a, b). The overall trend was the presence of *J. maritima* in the northwestern US and British Columbia (BC) with intermediate trees (hybrids and backcrosses) in eastern WA and OR, eastern BC and Kalispell, MT (Fig. 1). All the intermediate trees had *J. scopulorum* as the paternal parent (via pollen), with only two intermediate trees having *J. maritima* cp DNA (Wallowa, WO, Fig. 1). *Juniperus maritima* nrDNA (ITS) was found in all trees, except for two putative hybrids at Williams Lake, BC (WL, Fig. 1) and two hybrids at Fairmont Hot Springs, BC (FH, Fig. 1).

However, it was surprising to find no typical *J. scopulorum* (by all three DNA markers) in Wallowa, eastern Washington, or Kalispell, MT (Adams 2015b). Reference *J. scopulorum* trees from Utah and New Mexico were pure *J. scopulorum* (by the three DNA markers) (Fig. 1). Evidence was found of J. maritima introgression eastward into *J. scopulorum* in Montana, Wyoming, Idaho and Utah.

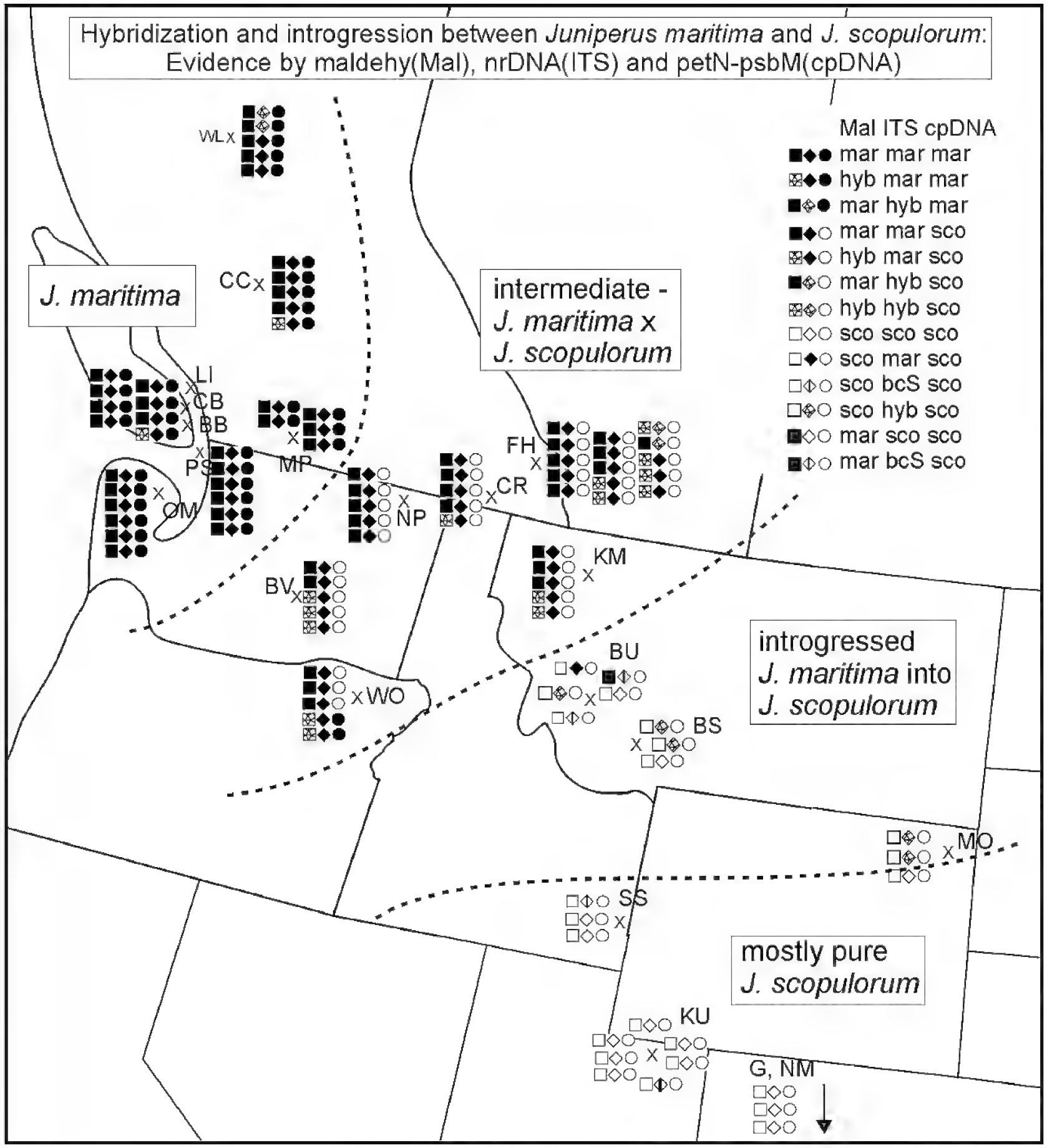

Figure 1. Combined mapping using three gene classifications. The study area can be divided into roughly four zones: 1. typical *J. maritima*: Puget Sound, Vancouver Island, islands in the Strait of Georgia, and western BC; 2. intermediate trees: eastern WA, Wallowa, OR, se BC, and western MT; 3. trees introgressed from *J. maritima* into *J. scopulorum*: Montana and ne Wyoming; 4. mostly typical *J. scopulorum*: se ID, Utah and south in the Rocky Mtns. Adapted from Adams 2015b.

Hybridization was discovered between *J. arizonica* (R.P. Adams) R.P. Adams and *J. coahuilensis* (Martínez) Gaussen ex R.P. Adams in southern New Mexico and trans-Pecos Texas using nrDNA and cp markers (Adams 2017). Introgression was found in the Hueco Tanks and Quitman Mtns. by pollen from *J. arizonica* (from the northwest) producing hybrids that were all paternal *J. arizonica* and maternal *J. coahuilensis*. Only one hybrid individual was found in the trans-Pecos (Alpine-Marfa-Ft. Davis area) and it had *J. coahuilensis* as its paternal parent. Considerable heterozygosity (in the nrDNA) appeared to be relictual from previous hybridization with unknown taxa.

The range of *Juniperus scopulorum* extends nearly to northern Mexico (Adams 2011b; 2014) and *J. blancoi* var. *mucronata* (R.P. Adams) Farjon ranges into northern Sonora and Chihuahua (Adams 2000, 2014). Individuals in Sonora and western Chihuahua are thought to be intermediate between *J. scopulorum* and *J. b.* var. *mucronata* (Adams 2014). Therefore, it seemed timely to apply molecular methods to determine the extent (if any) of gene exchange between these taxa. The purpose of the present paper is to report on extensive analyses of nrDNA (ITS) and cp DNA (petN-psbM) of populations of *J. blancoi* Martínez (and its varieties) in northern Mexico and *J. scopulorum* in the southwestern USA with emphasis on determining hybridization and introgression.

It should be noted that analysis of the distribution of cp types is very useful in the analysis of hybridization and introgression due to the uniparental inheritance of chloroplasts in conifers (see Adams 2019 for a recent review). In the Cupressaceae, chloroplasts have been reported to be paternally (pollen) inherited in all species, except *Cunninghamia konshii* and 2 out of 6 plants examined in the intergeneric cross (*Callitropsis nootkatensis* x *Hesperocyparis macrocarpa*) that were maternally inherited (Adams 2019). Although, at present, no study has documented the inheritance of cp in *Juniperus*, Adams et al. (2016) found cp are paternally (via pollen) inherited in a cross between *Hesperocyparis* (= *Cupressus*) *arizonica* x *H. macrocarpa*). Because *Hesperocyparis* is a sister genus to *Juniperus* (Adams 2014), it seems likely that inheritance of cp via pollen (e.g., paternal) in *Juniperus* is the same as for *Hesperocyparis* and other Cupressaceae species.

MATERIALS AND METHODS

Plant material: (species, population acronym, location, vouchers):

Juniperus blancoi var. *blancoi*:

Carmona - El Oro area, central México

***Adams 6849, 6850*, 6851**, stream-side, 7-8 km south of Carmona, 19° 45'N, 100° 07' W, 2580 m, 15 Dec 1991, Mexico, MX.

El Salto, Durango

Adams 10257, 10258, with S. Gonzalez and M. Gonzalez-Elizondo, dioecious, tree-shrub, 1.5 m tall, with 1 main stem, but damaged by flood water so it is branched, foliage lax or pendulous, ultimate branchlets generally planate; bark brown, exfoliating in thin plates, reddish beneath, branchlets ~1cm dia. scaly with bronze color beneath scales; female, past pollination. mature fruit with 2 lobes or globose (if one seeded), dark blue upon maturity. On the banks of a running stream approx. 7 km from El Salto town square on road to Pueblo Nuevo, 23° 45.241'N, 105° 22.851' W, 2580 m, 9 May 2004, Durango, MX

J. b. var. *huehuentensis* R.P. Adams, S. González & M. González:

Cerro Huehuento, Durango

Adams 10247-10249, with S. Gonzalez and M. Gonzalez Elizondo, dioecious, shrub, 0.5 m x 2 m wide, foliage yellowish green, common on rock at top of Cerro Huehuento. brown bark exfoliating in thin plates. most female cones bi-lobed, dark blue when ripe (1 yr.), female cones on the underside of planate branches. pollen shedding now (just finishing), (mid-March - mid-May). 24° 04.587'N, 105° 44.463' W, 3227 m, 8 May 8 2004, Durango, MX

Cerro Mohinora, Chihuahua

***Lab acc. Adams 11436**, ex Socorro Gonzalez 7348a,* prostrate shrub 0.3 m tall, strong lime aroma, on rock almost atop Cerro Mohinora, 25° 57' 22" N, 107° 02' 51" W, 3303 m, 2 Nov 2007, Chihuahua, MX.

***Lab acc. Adams 11916, 11917, 11918**, ex Socorro Gonzalez 7531a, b, c* with D. Ramírez, A. Mastretta and S. Tena, shrub 0.5-1 m, decumbent, branches olive green color, on rock almost atop Cerro Mohinora, 25° 57' 34" N, 107° 02' 58" W, 3160 m, 2 June 2009, Chihuahua, MX.

J. b. var. *mucronata*:
Yepachic area, Chihuahua/ Sonora
Adams 8701-8702, trees to 8 m, with strong central axis, foliage lax at tips, bark exfoliating in strips, fruits reniform (bilobed) and globose, with oaks, *Juniperus deppeana, Pinus*, on Mex. Highway 16 between km 336-337, 10 km east of Yepáchi, Chih., 14 km west of Sipachic, Chia., on road to Yecora, Sonora, 28˚ 26' 32" N, 108˚ 28' 39" W, 1530 m, 20 Dec 1998, Chihuahua, MX.
Adams 8703, trees to 8m, with strong central axis, wood bright purple, foliage weeping at tips, leaf tips mucronate, bark exfoliating in strips, with Oaks. common along the south banks of the Puente El Talayote, 19 km west of Maicoba on Mex. Highway 16 at km 307, on road to Yecora, Son. 28˚ 22' 29" N 108˚ 45' 46" W, 1180m, 20 Dec.1998, Sonora, Mexico
Mesa Tres Ríos - Río Gavilán area, Sonora
Lab acc. Adams 15548, *ex George M. Ferguson 4390,* N-facing slope, female tree 8.5 m tall, no cones, bright green 29˚ 48' 51.8" N, 108˚ 44' 7.8" W, 1745 m, 7 Aug 2018, Arroyo El Palmilloso (=Rio La Cueva), Nácori Chico, Sierra Madre Occidental, Sonora, MX.
Colonia Pacheco, Chihuahua (near Mesa Tres Rios, so included in that group in text, table and figures)
Adams 2512, growing next to flowing stream on rocks, with 2-lobed fruits as in *J. blancoi*, with pines and oaks on flatlands, lots of little junipers under oaks with *J. deppeana*, 5 km E of Colonia Pacheco, on Rio Piedras Verde, 30˚ 05' 23.8" N, 108˚ 20' 19" W, 1980 m, 20 Feb 1978, Colonia Pacheco, Chihuahua, MX.
Lab acc. Adams 15549, *ex George M. Ferguson 4401,* N-facing slope, female tree 20 cm dbh, 7 m tall; numerous cones dark blue, Arroyo San Antonio, 4.8 km (by road) N Mesa Tres Rios, 29˚ 52' 1.9" N, 108 ˚ 42' 41.0" W, 1705 m, 8 Aug 2018, Nácori Chico, Sierra Madre Occidental, Sonora, MX.
Lab Acc. ***Adams 15550****, ex George M. Ferguson, 4404*, male tree 62 cm dbh, 12 m tall, pollen cones dry (shedding completed), bark longitudinally furrowed, Arroyo San Antonio, 6.4 km (by road) N Mesa Tres Rios, 29˚ 52' 35.4" N, 108˚ 43' 15.2" W, 1570 m, 8 Aug 2018, Nácori Chico, Sierra Madre Occidental, Sonora, MX.
Lab Acc. ***Adams 15551, 15552****, ex. George M. Ferguson 4434, 4435,* trees, 5-8 m tall; bark longitudinally plated, confluence Rio Gavilán and Rio La Cueva, 29˚ 52' 58.4" N, 108˚ 37' 36.5" W, 1400 m, 10 Aug 2018. Nácori Chico, Sierra Madre Occidental, at Sonora, MX.
Los Ajos, Sonora
Adams 14424, 14425, 14426*, ex George M. Ferguson 3651,3652,3653,* trees to 8 m, bark plated dark brown. Riparian in pine-oak-juniper woodland w/ *Juniperus deppeana, Acer grandidentatum, Alnus oblongifolia, Quercus arizonica, Pinus chihuahuana, Pinus engelmannii, Platanus wrightii*. Municipio Cananea, Sierra Los Ajos, Canon Evans, 1.5 mi NW (by road) Los Ajos Nuevos CONANP headquarters. 30˚ 59' 14.8" N, 109˚ 58' 22.8" W, 1800 m, 26 Aug 2014, Sonora, MX.
J. scopulorum:
Guadalupe Mtns., NM
Lab acc. ***Adams 15602***, *ex Richard Worthington 28617,* UTEP Herbarium accession 58749, Devil's Den Spring, Guadalupe Mtns. 32° 02' 3.12" N, 104° 16' 0.12" W. 2103 m (6000ft), 5 Sept. 1999, Eddy Co., NM.
Lab Acc. Adams 15603,
ex Richard Worthington 28673, UTEP Herbarium accession 58750, North Fork, Big Canyon, Guadalupe Mtns. 32° 02' 3.12" N, 104° 45' 0.12" W. 1828m (6000ft), 6 Sept. 1999, Eddy Co., NM.
Lab acc. ***Adams 15783, 15784, 15785***, *ex George M. Ferguson 4624, 4625, 4626* with J. Ferguson, 3-9 m tall; bark longitudinally plated, pollen cones forming, riparian woodland, limestone, associated species: *Pinus ponderosa, Pinus edulis, Juniperus deppeana, Quercus muehlenbergii, Quercus grisea, Acer grandidentatum, Berberis haematocarpa, Arbutus xalapensis, Dasylirion leiophyllum, Agave parryi*, Dark Canyon, S of Klondike Gap near confluence Hooper Canyon, 0.2 mi (by FR 307) E jct County Road 412 (FR 69), just inside USFS boundary. Lincoln National Forest, Guadalupe Mountains, 32˚ 6' 0" N, 104˚ 46' 15.6" W, 1920 m (6300 ft.), 3 Nov 2019 Eddy County, NM**.**

***Lab Acc. Adams 15786**, ex George M. Ferguson 4627* with J. Ferguson, male tree, ca. 15 cm dbh, 4 m tall; bark longitudinally plated, associated species: *Pinus ponderosa, Pinus edulis, Juniperus deppeana, Quercus muehlenbergii, Quercus grisea, Acer grandidentatum, Berberis haematocarpa, Arbutus xalapensis, Dasylirion leiophyllum, Agave parryi*, riparian woodland, limestone, Dark Canyon, S of Klondike Gap near confluence Hooper Canyon, 0.2 mi (by FR 307) E jct County Road 412 (FR 69), just inside USFS boundary, Lincoln National Forest, Guadalupe Mountains, 32° 6' 0" N, 104° 46' 15.6" W, 1920 m (6300 ft.), 3 Nov 2019 Eddy County, NM**.** *Juniperus scopulorum* hybrid ITS x *J. blancoi*
Black Canyon, Gila NF, NM

Lab acc. ***Adams 15562, 15563***, *ex George M. Ferguson 4279, 4280,* 8 m tall, foliage weeping, Gila National Forest, East Fork Gila River, 0.5 mi below confluence with Black Canyon, 33° 10' 45.1" N, 108° 9' 32.0" W, 1743 m (5720 ft.), 28 July 2018, Grant Co., NM.

Sapillo Creek, Gila National Forest, NM

Lab acc. ***Adams15714, 15715, 15716, 15717***, *ex George M. Ferguson 4549, 4550, 4551, 4552*, male tree, 4 m tall, bark longitudinally plated, Pine forest, associated species: *Pinus ponderosa var. brachyptera, Pinus edulis, Juniperus deppeana,* Gila National Forest, Sapillo Creek (Gila River drainage), 1.0 mi (by NM hwy 35) E Upper End Campground of Lake Roberts, 33° 01' 34.3" N, 108° 08' 8.6" W, 1875 m (6150 ft), 4 July 2019. Grant Co., NM.

Gila Hot Springs, Gila National Forest, NM

Lab acc. ***Adams15718, 15719, 15720*** *ex George M. Ferguson 4553, 4554, 4555* trees to 10 m tall, Pine forest, bark longitudinally plated, with: *Pinus ponderosa var. brachyptera, Pinus edulis, Juniperus deppeana, Juniperus monosperma,* Gila Hot Springs, on private land along W Fork Gila River, 33° 11' 44.5" N, 108° 12' 25.9" W, 1707 m (5600 ft), 5 July 2019. Grant Co. NM

Lab acc. ***Adams 15722, 15723,*** *ex George M. Ferguson 4557, 4558,* Pine forest; trees, 8 m tall, bark longitudinally plated, with: *Pinus ponderosa var. brachyptera, Pinus edulis, Juniperus monosperma.* Gila National Forest, near Woody's Corral, 0.5 mi (by NM hwy 15) E Gila Cliff Dwellings National Monument, along West Fork of Gila River. 33° 13' 30.2" N, 108° 15' 05.2" W, 1707 m,(5650 ft), 5 July 2019, Catron Co., NM

Sedona, AZ

Adams 10637-10639, Common at top of canyon, trees to 6 m, with *J. deppeana*. On AZ highway 89A, between Sedona and Flagstaff in Oak Creek Canyon. 34° 57' 19" N 111° 45' 17" W, 1521 m, 13 March 2005, Yavapai Co., AZ

Brian Head, UT

Adams 10908-10910, female, 6 m tree with strong central axis, common with pinyon, sage and oak on gravelly soil, approx. 10 km SE of Parowan, near Brian Head on UT hwy 148, 37° 45' 09" N, 112° 50' 19" W, 2414 m, 6 Aug 2005, Iron Co., UT.

Lukachukai, AZ

Adams 10915-10917, 4-6 m tall, strong central axis, very glaucous foliage, with Ponderosa pine and oaks on rocky soil. Chuska Mts., 14 km NE of Lukachukai, AZ on AZ hwy. 13. 36° 27' 14" N, 109° 09' 48" W, 2409 m, 7 Aug 2005, Apache Co., AZ

Eagar, AZ

Adams 10928-10930, 10-12 m trees, strong central axis, foliage very glaucous and weeping, on rocky soil, scattered with Ponderosa pine, spruce and *J. deppeana*, *J. monosperma* and *J. communis* on Water Canyon Rd., 6 km S. of Eagar, AZ., White Pine Mts., 34° 02' 41" N, 109° 17' 52" W, 2441 m, Aug 8, 2005, Apache Co., AZ.

Glorieta, NM

Adams 10933-10935, 5-7 m trees, strong central axis, very glaucous foliage, abundant with *J. monosperma*, Glorieta Mesa, on I25, E of Santa Fe, Exit 297 (Valencia), 35° 34' 44" N, 105° 47' 35" W, 2256 m, 9 Aug 2005 m, Santa Fe Co., NM.

Voucher specimens are deposited in the Herbarium, Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM), (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized.

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

Sequencing of petN-psbM (cp DNA) proved be useful in distinguishing *J. blancoi* from *J. scopulorum* by the presence of a 5 bp indel at site[1] 703 (TTTTA insert) in *J. blancoi*. The presence of the insert was found in *J. blancoi* from Carmona, and El Salto, as well as all *J. b.* var. *huehuentensis* from Cerro Huehuento (Table 1). However, in the Cerro Mohinora population of *J. b.* var. *huehuentensis,* one plant (11917) contained the *J. scopulorum* cp type (Table 1). Further north, all the *J. b.* var. *mucronata* populations (Yepachic, Colonia Pacheco, Mesa Tres Rios, Los Ajos) contained only the *J. scopulorum* cp type. This supports the hybrid origin of *J. b.* var. *mucronata* from *J. blancoi* and *J. scopulorum* hybridization in the past. In contrast, no plants collected as *J. scopulorum* contained the *blancoi* cp type (Table 1).

DNA sequencing of nrDNA (ITS) yielded 1270bp, with three informative sites (284[2], 346[3], 348[4]) that distinguished *J. scopulorum* and *J. blancoi* (all varieties) (Table 1). Several *J. b.* var. *mucronata* trees in northern Mexico had polymorphic sites indicative of present or past hybridization with unknown taxa (Table 1). Four *J. scopulorum* trees appear to be of hybrid origin (15786, 15803, Guadalupe Mtns., 19015, 10916, Lukachukai, AZ), although all had the *J. scopulorum* cp type.

Mapping samples using nrDNA types (Fig. 2) reveals that hybrids (by nrDNA) are found in northern Mexico in *J. b.* var. *mucronata*; and two hybrids at Lukachukai, AZ, (Fig. 2). Two backcrossed plants were found in the Mesa Tres Rios population and two in the *J. scopulorum* site in the Guadalupe Mtns. (Fig. 2).

Overall, the variation can be divided into three zones (Fig. 2): northern zone - mostly pure *J. scopulorum*; central zone that contains hybrids and introgressed *J. b.* var. *mucronata* that have only the *J. scopulorum* cp; and zone 3: mostly pure *J. blancoi* and *J. b.* var. *huehuentensis*. An interesting trend is that in the central (red) zone, all samples have the *J. scopulorum* cp (Fig. 2). However, the nrDNA varies from 'pure' J. blancoi', to hybrids (*J. scopulorum* x *J. b.* var. *mucronata*), to 2 backcrossed plants with 2 nrDNA homozygous *J. blancoi* sites and one site each, heterozygous *J. blancoi, J. scopulorum*. (Table 1, Fig. 2). Two plants from Guadalupe Mtns., NM have nrDNA suggesting they are backcrosses (Table 1).

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[1]Informative intel in petN-psbM at site 703: xTTTTATAGTAA, where x = TTTTA
Informative nrDNA (ITS) sites: [2]284 xCCCGCGGTGC; [3]346 GAAACGACx; [4]348 xTGTGCGGA.

A detailed examination of the cp types, shows (Fig. 2) all the var. *mucronata*, and one var. *huehuentensis* had *J. scopulorum* cp (via a paternal, pollen parent), but no *J. scopulorum* contained the *J. blancoi* cp DNA (i.e., via pollen from *J. blancoi*). Gene flow via pollen is decidedly unidirectional from *J. scopulorum* to *J. blancoi*, north to south. Unidirectional hybridization has been reported when pollen of *Pinus brutia* was placed on ovulate cones of *P. halepensis,* no viable seeds were found, but the reciprocal cross (pollen, *P. halepensis* x ovulate cones, *P. brutia*) yielded viable seeds (Conkle et al. 1988; Moulalis et al. 1976). The occurrence of two nrDNA hybrids in the Lukachukai population is anomalous. There is no indication of pollen flow northward into *J. scopulorum* in this region.

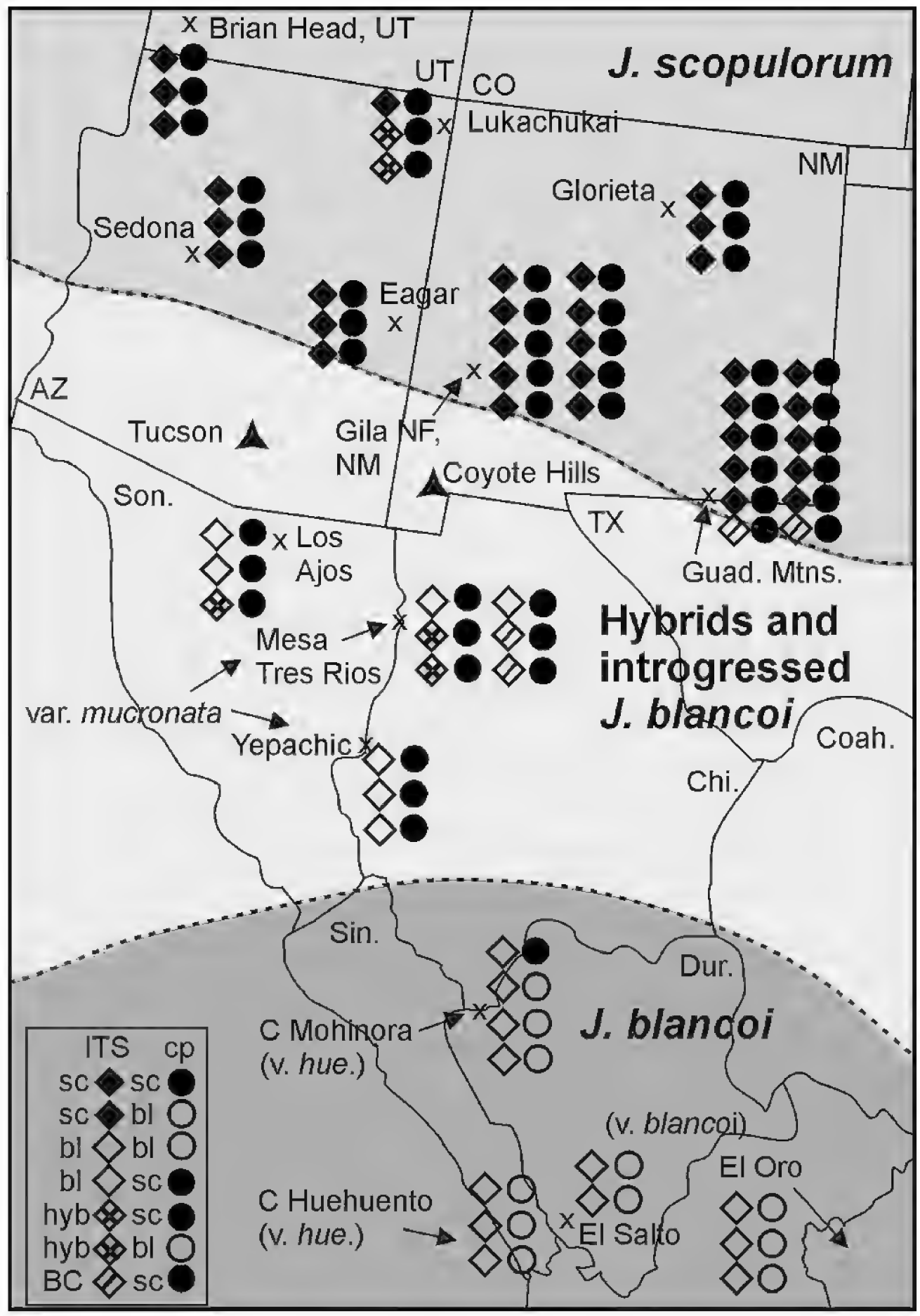

Figure 2. Distribution of cp and nrDNA types in three zones: mostly pure *J. blancoi* (blue), mostly pure *J. scopulorum* (yellow) and a zone of hybrids and introgressants (red). Combinations of cp and nrDNA shown in box, lower left. See text for discussion.

Pollen is shed by *J. scopulorum* in March-April and *J. blancoi* in January- mid-May (Adams 2014), although local differences and yearly differences in pollen shedding times are well known (Levetin 1998). Analysis of wind directions and velocities show that the predominant directions in January through April are from the southeast, but 6 to 10% of the winds are from the northwest (Fig. 3). The winds from the northwest are mostly 7.4 - 11.2 mph and 11.2 - 19.9 mph (Fig. 3). The distance from Sedona to Los Ajos is 290 miles and from Eager to Mesa Tres Rios, 290 miles. If northwest *J. scopulorum* pollen was carried by winds of 10 mph, it would take only 29 hrs. to reach Los Ajos or Mesa Tres Rios.

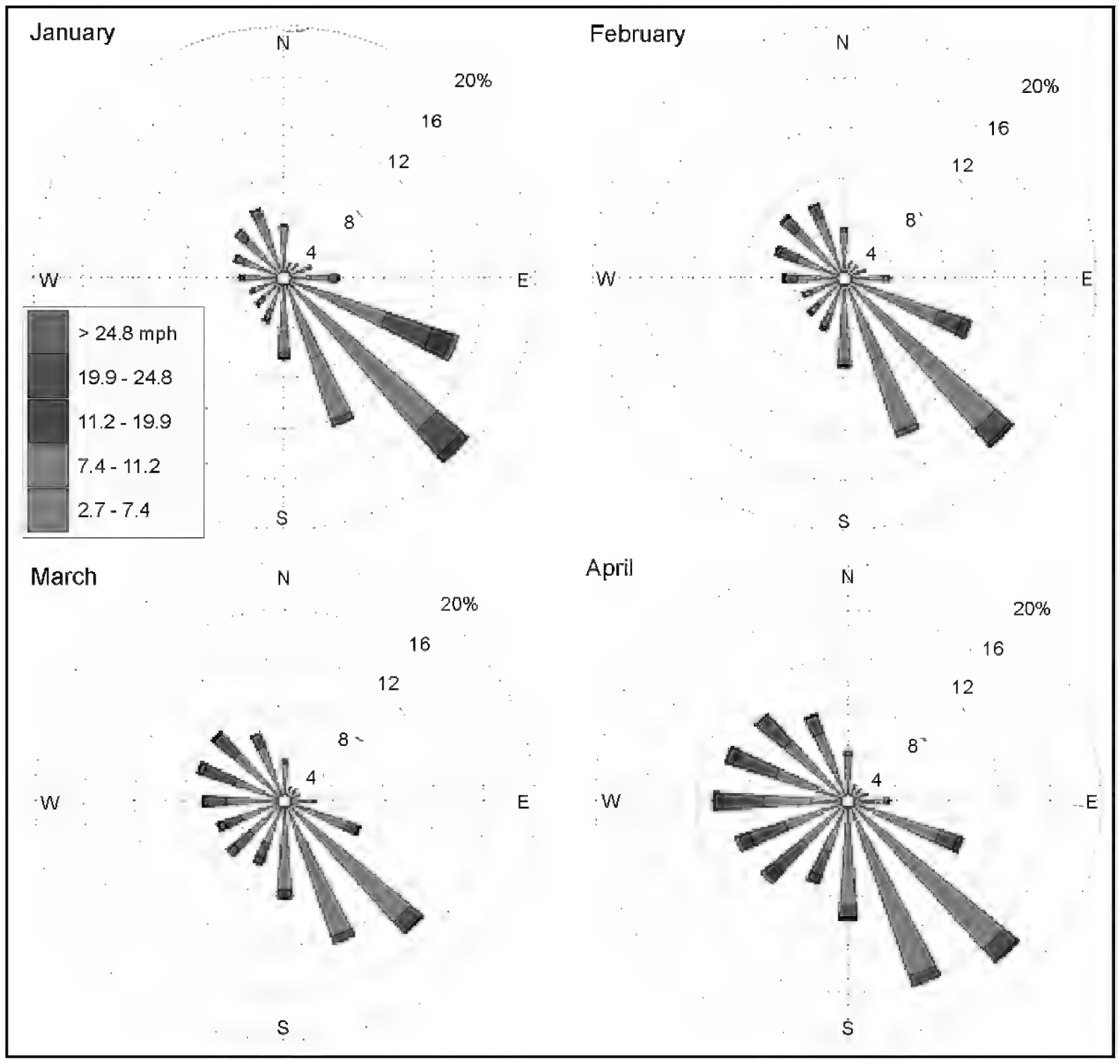

Figure 3. Frequencies of wind directions and velocities in January - April, in Tucson, AZ. Data from Wind Rose Resources, NRCS, https://www.wcc.nrcs.usda.gov/climate/windrose.html

Several other studies in conifers have reported long distance transport (LDT) of pollen from a few km to several hundred km (Szczepanek et al. 2017; Neale and Wheeler 2019; Stewart et al. 2012; Sarvas 1962; Koski 1970; Nichols et al. 1978; Campbell et al. 1999). Importantly, several studies have reported that LDT pollen has maintained its viability (Lindgren et al. 1995; Varis et al. 2009; Williams 2010). Pollen from *Juniperus communis*, in the western Alps, was stored at ambient conditions and found to be 40-90% viable for fresh pollen, 20-40% viable after two weeks and 0-10% viable after two months storage (Carmeliello et al. 1990). Finally, it should be mentioned that in a preliminary study on LDT pollen viability, Levetin (per. comm.) found viable *Juniperus* (*J. ashei*) LDT airborne pollen in Tulsa, OK, after having traveled at least 200 mi. from southern Oklahoma/Texas.

Thus, because the *J. scopulorum* pollen from southern Arizona and New Mexico can easily reach Los Ajos, Mesa Tres Rios and beyond, in 1 to 2 days, it seems likely the pollen would be viable to affect fertilization. The predominant southeast winds (Fig. 3), blowing pollen from northern Mexico towards the northwest favor the movement of pollen in March-April towards Sedona, Edgar and Gila National Forest. Yet, we have discovered no trees containing the *J. blancoi* cp type from there (Table 1, Fig. 2). It appears that *J. scopulorum*, in present times, has an efficient sterility barrier against fertilization by *J. blancoi* pollen. However, *J. b.* var. *mucronata* seems to have an ineffective barrier against *J. scopulorum* pollen fertilization as indicated by the fact that all the var. *mucronata* plants sampled have the *J. scopulorum* cp type (Table 1, Fig. 2).

The hybrids near Lukachukai (2400 m) and in the Guadalupe Mtns. (1900-2200 m) have maternal *J. blancoi* parents, but are far from any known *J. blancoi* trees (Fig. 2). Martin and Mehringer (1965) and Wells (1966) suggested woodland and forest species descended as much as 800m throughout the southwest from 13,500 to 10,000 ybp. A review of late Pleistocene- Holocene climate in Mexico (Metcalfe, et al. 2000) noted that during the late Pleistocene and early Holocene, northern Mexico was much wetter and cooler than today, with Pinyon-Juniper woodland covering extensive areas which are, today, desertshrub.

Juniperus scopulorum dated in the late Pleistocene (13,830 ybp), has been found in packrat middens at ~1500m at Coyote Hills (Fig. 2), southwest New Mexico (Holmgren et al. 2003) in an area that is, presently, a Chihuahuan desert grassland with scattered *J. arizonica* trees (GMF, pers. obs.).

Holmgren et al. (2003) noted that *J. scopulorum* was common in Pleistocene middens at ~1500m, on limestone at Guadalupe and Sacramento Mtns. sites (Van Devender et al. 1984). If *J. scopulorum* grew at 1555m (Sacramento Mtns midden) and 1500m (Guadalupe Mtns. midden) that would be about 500m lower than present *J.* scopulorum plants in those areas. In addition, an isolated occurrence of *J. scopulorum* has been reported at 975m in Pleistocene middens from Organ Pipe National Monument on the Arizona - Mexico border (Van Devender, 1990). Van Devender (1990), noted that the nearest present-day population of *J. scopulorum* is 270 km northeast, below the Mogollon Rim, AZ. During the late Pleistocene, *J. scopulorum* and *J. blancoi* var. *mucronata*, both of which favor riparian or streamside habitats today, could have expanded their ranges such that the taxa overlapped (sympatric) in many areas, giving opportunities to hybridize. A northern origin of *J. blancoi* was hypothesized by Mastretta-Yanez et al. (2012), who found the most ancestral haplotype for the species in the Yecora population of *J. blancoi* var. *mucronata*. Their ancestral cp haplotype is surely the *J. scopulorum* cp we have found in **every** *J. b.* var. *mucronata* plant analyzed in this study (Fig. 2). And, this seems to agree with the present phylogeny of the smooth leaf junipers of North America (Fig. 1.16.5, p. 22, Adams 2014) showing *J. blancoi* var. *mucronata* linked to *J. scopulorum*.

nrDNA is well known to maintain heterozygosity acquired millions of years ago (see Syring et al. 2007). In fact, Syring et al. (2007) estimated that in *Pinus*, it may take up to 76 myr to achieve complete genome wide coalescence by concerted evolution (Aguilar et al. 1999; Liao 1999; Moreno-Letelier et al.

2014). So, it shouldn't be surprising to find ancestral polymorphisms in extant populations today. With the retreat of the Wisconsin glacial ice, and the subsequent warming (Crandell 1971; Flint 1971), *Juniperus* expanded its range into the higher elevation habitats that it occupies today. The expanded and overlapping *Juniperus* ranges help explain the relictual hybridization between *J. blancoi* and *J. scopulorum* that we found in northern Mexico in this study. Alternatively, pollen of J. scopulorum carried south into the range of *J. b.* var. *mucronata*, and affecting pollination may have produced the introgressed taxon.

Of course, it is possible that *J. blancoi* var. *mucronata* (in the red, central zone) has acquired the *J. scopulorum* cp by chloroplast capture as an ancient event. If so, this study may have captured a time interval catching a snapshot of *J. b.* var. *mucronata* in the midst of a chloroplast capture event. Chloroplast capture in *Juniperus* has been reported from an ancestor of *J. sabina* var. *balkanensis* from *J. thurifera* (Adams et al. 2017, Adams et al. 2018). Presumedly, the nrDNA has become homogenous for the *J. sabina* type due to concerted evolution, however, the *J. thurifera* cp has been retained. Examination of Fig. 2 shows that indeed, all plants of *J. b.* var. *mucronata* contain the *J. scopulorum* cp, and about half the samples have homozygous *blancoi* nrDNA, and the rest have hybrid, heterozygous nrDNA. Without additional gene sequences, it does not seem possible to make a definitive decision. Analyses of more genes (NextGen sequencing) will undoubtedly reveal a more complete picture of the evolutionary chloroplast capture events highlighted in the present report.

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Table 1. Analyses of *J. blancoi* and *J. scopulorum* by cpDNA (petN-psbM): indel site 703[1], and nrDNA: informative sites 284, 346 and 348.BI intgr Sc cp = blancoi, introgressed by scop cp; BxS, BC-BI = blancoi x scop, backcrossed to blancoi. SxB, BC-Sc = scop x blancoi, backcrossed to scopulorum.

collection number, taxon (field identification), population	id by cpDNA	id by nrDNA	284(204) T/C(Y)	346(266) T/G(K)	348(268) T/C(Y)
10908 scopulorum Brian Head, UT	scop	scop	T	T	T
10909 scopulorum Brian Head, UT	scop	scop	T	T	T
10910 scopulorum Brian Head, UT	scop	scop	T	T	T
10933 scopulorum Glorieta NM	scop	scop	T	T	T
10934 scopulorum Glorieta NM	scop	scop	T	T	T
10935 scopulorum Glorieta NM	scop	scop	T	T	T
10917 scopulorum Lukachukai, AZ	scop	scop	T	T	T
10928 scopulorum Eagar, AZ	scop	scop	T	T	T
10929 scopulorum Eagar, AZ	scop	scop	T	T	T
10930 scopulorum Eagar, AZ	scop	scop	T	T	T
10637 scopulorum Sedona, AZ	scop	scop	T	T	T
10638 scopulorum Sedona, AZ	scop	scop	T	T	T
10639 scopulorum Sedona, AZ	scop	scop	T	T	T
15562 scopulorum Black Canyon, Gila NF NM	scop	scop	T	T	T
15563 scopulorum Black Canyon, Gila NF NM	scop	scop	T	T	T
15714 scopulorum Sapillo Ck., Gila NF, NM	scop	scop	T	T	T
15715 scopulorum Sapillo Ck., Gila NF, NM	scop	scop	T	T	T
15716 scopulorum Sapillo Ck., Gila NF, NM	scop	scop	T	T	T
15718 scopulorum Gila Hot Sprs, Gila NF, NM	scop	scop	T	T	T
15719 scopulorum Gila Hot Sprs, Gila NF, NM	scop	scop	T	T	T
15720 scopulorum Gila Hot Sprs, Gila NF, NM	scop	scop	T	T	T
15722 scopulorum Woodys Corral, Gila NF NM	scop	scop	T	T	T
15723 scopulorum Woodys Corral, Gila NF NM	scop	scop	T	T	T
15602 scopulorum, Guadalupe Mtns, NM	scop	scop	T	T	T
15603 scopulorum, Guadalupe Mtns, NM	scop	scop	T	T	T
15783 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15784 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15785 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15798 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15799 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15800 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15801 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15802 scopulorum Guadalupe Mtns, NM	scop	scop	T	T	T
15786 scopulorum, Guadalupe Mtns, NM	scop	SxB, BC-Sc	T	T/G (K)	T/C (Y)
15803 scopulorum, Guadalupe Mtns, NM	scop	SxB, BC-Sc	T	T/G (K)	T/C (Y)
10915 scopulorum Lukachukai, AZ	scop	hybrid	T/C (Y)	T/G (K)	T/C (Y)
10916 scopulorum Lukachukai, AZ	scop	hybrid	T/C (Y)	T/G (K)	T/C (Y)
15552 blancoi var. mucronata, Mesa Tres Rios	scop	hybrid	T/C (Y)	T/G (K)	T/C (Y)
15548 blancoi var. mucronata, Mesa Tres Rios	scop	hybrid	T/C (Y)	T/G (K)	T/C (Y)
14425 blancoi var. mucronata, Los Ajos	scop	hybrid	T/C (Y)	T/G (K)	T/C (Y)
15551 blancoi var. mucronata, Mesa Tres Rios	scop	BxS, BC-BI	C	G	T/C (Y)
15549 blancoi var. mucronata, Mesa Tres Rios	scop	BxS, BC-BI	T/C (Y)	G	C
11917 blancoi var. huehuentensis, Cerro Mohinora	scop	blancoi	C	G	C
8701 blancoi var. mucronata, Yepachic	scop	blancoi	C	G	C
8702 blancoi var. mucronata, Yepachic	scop	blancoi	C	G	C
8703 blancoi var. mucronata, Yepachic	scop	blancoi	C	G	C
14424 blancoi var. mucronata, Los Ajos	scop	blancoi	C	G	C
14426 blancoi var. mucronata, Los Ajos	scop	blancoi	C	G	C
2512 blancoi var. mucronata, Col. Pacheco (MTRios)	scop	blancoi	C	G	C
15550 blancoi var. mucronata, Mesa Tres Rios	scop	blancoi	C	G	C
11436 blancoi var. huehuentensis, Cerro Mohinora	blancoi	blancoi	C	G	C
11916 blancoi var. huehuentensis, Cerro Mohinora	blancoi	blancoi	C	G	C
11918 blancoi var. huehuentensis, Cerro Mohinora	blancoi	blancoi	C	G	C
10247 blancoi var. huehuentensis, Cerro Huehuento	blancoi	blancoi	C	G	C
10248 blancoi var. huehuentensis, Cerro Huehuento	blancoi	blancoi	C	G	C
10249 blancoi var. huehuentensis, Cerro Huehuento	blancoi	blancoi	C	G	C
10257 blancoi, El Salto	blancoi	blancoi	C	G	C
10258 blancoi, El Salto	blancoi	blancoi	C	G	C
6849 blancoi, Carmona - El Oro	blancoi	blancoi	C	G	C
6850 blancoi, Carmona - El Oro	blancoi	blancoi	C	G	C
6851 blancoi, Carmona - El Oro	blancoi	blancoi	C	G	C

Examining Ecological Characteristics of Populations of *Acer grandidentatum* Nutt. (Aceraceae, bigtooth maple) in Central Texas

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ABSTRACT

Part of the central Texas Edwards Plateau physiographic area was examined using satellite and drone photographs taken in autumn prior to leaf fall. This was done to identify and locate deciduous communities prior to ground truthing to identify overstory and understory woody species. Using satellite images, potential sites were identified for more careful examination. A drone was next flown over the potential sites and identified 26 most promising sites covering 174 ha. Based on community size and ease of access, nine sites were ground surveyed, eight with the quadrat procedure and the ninth visually. The mean of the deciduous communities was 1.2 ha (range=0.3-4.5 ha) surrounded by *Juniperus ashei* woodlands. All but one was in a steep limestone canyon. Seventeen overstory woody species were found in these deciduous plant communities. Mean overstory density was 559 plants/ha and mean basal area 25.5 m^2/ ha. Three species are relatively rare in central Texas including *Acer grandidentatum* Nutt. (Aceraceae, bigtooth maple, relative density 30%, relative basal area 38% in these communities). *Quercus muehlenbergii* Engelm. (Fagaceae, chinquapin oak) and *Tilia caroliniana* Mill. (Tiliacae, Carolina basswood), were also present but lower in both values. Thirty understory woody species were found with *Celtis laevigata* Willd. (Ulmaceae, Texas sugarberry) and *Q. laceyi* (Laceyi oak) having the highest densities. Recruitment into the adult population by juveniles of some of these overstory species seemed minimal or lacking because of low density. These communities were found in reduced light in steep limestone canyons, especially the north-south canyons and on deeper soils that could hold more water than the shallow upland soils. *Published on-line www.phytologia.org* ***Phytologia*** *102(2): 27-40 (June 24, 2020). ISSN 030319430.*

KEY WORDS: populations; deciduous communities; rare species; overstory; understory; satellite photographs; drone; limestone canyons; recruitment.

A large part of Central Texas is called the Edwards Plateau physiographic region; it is not a plant community, but a large, heavily dissected, calcareous plateau (Hill 1892; Bray 1904; Tharp 1939; Gould 1962; Correll and Johnston 1979). It consists of about 9.7 X 10^6 ha or approximately 1 X 10^5 km^2. There are many different plant communities within this area including various grasslands, savannas, woodlands and forests (Amos and Gehlbach 1988; Gehlbach 1988; Riskind and Diamond 1988; Diamond et al. 1995; Van Auken 2018). The presence or unusual plants in this central Texas area were noted by early travelers and

explorers (see Inglis 1962; Weniger 1988). The woody plants, specifically the timber was mentioned quite early (Bray 1904) as was the unusual flora present in some of these central Texas canyon communities (Palmer 1920). However, community structure and factors that seemed to promote or control the deciduous communities has only been examined recently (Van Auken et al. 1981; Nelson-Dickerson and Van Auken 2016; Van Auken et al. 2017).

The vast majority of the area in this central Texas Edwards Plateau physiographic region is private property and not well studied. Many of the private properties are fairly large with steep-sided hills and deep limestone canyons. Usually when these properties change hands the management strategy changes as well (Carpenter and Brandimarte 2014). Thus, it is very difficult to determine the plant communities of the past or what will happen in the future. In the past, domestic grazing and timber cutting were the main industries of the general area. Because of a reduction in the amount of grassland and savanna, caused by constant heavy grazing and the removal of light fluffy fuel by the cattle grazing, fire frequency was dramatically reduced promoting increased density of woody plants especially *Prosopis* (mesquite) and *Juniperus* (mountain cedar). Timber harvest has also been considerably reduced because of over harvest and slow re-growth (Riskind and Diamond 1988).

Some of these deciduous woodland communities have been shown to have populations of *Prunus serotina* (black cherry), *Quercus laceyi* (Lacey oak), *Q. buckleyi* (Texas red oak), *Aesculus pavia* (red buckeye) and other mostly deciduous species (Van Auken et al.1981). While others have populations of *Acer grandidentatum* (bigtooth maple, Nelson-Dickerson and Van Auken 2016; Van Auken and Taylor 2017; Van Auken et al. 2017). These later studies examined the structure and potential population changes of *A. grandidentatum* in two State Natural Areas. Thus, *A. grandidentatum* populations in central Texas have been studied more carefully for the past few years, but only in protected areas.

Many juvenile woody plants of this area are known to be sensitive to herbivory (Russell and Fowler 1999, 2002, 2004; Nelson-Dickerson and Van Auken 2016) and they appear to be plants capable of completing the early part of their life cycle in shade below a woodland canopy (Nelson-Dickerson and Van Auken 2016). These plants have slow growth in low light conditions. The niche of some of these deciduous woody plants seems to be deep, sheltered, limestone canyons or steep, north facing slopes. Some of these plants, in central Texas canyons, established approximately 300 years ago (Van Auken et al. 2017). However, only a few species in some of these deciduous communities have been carefully examined, and the cause of variation in community composition is mostly speculation.

Acer grandidentatum the main focus of this study is fairly widespread in the mountains of the southwestern United States, including mountain ranges in New Mexico and western Texas (Little 1944; Rice 1960; Hanks and Dick-Peddie 1974; Dent and Adams 1983; Alexander et al. 1984; Tollefson 2006; USDA-Plants 2019). However, isolated populations of *A. grandidentatum* are found in central Texas (Gehlbach and Gardner 1983; Nelson-Dickerson and Van Auken 2016) and in central and western Oklahoma (Rice 1960). *Acer grandidentatum* and some of the associated species in these deciduous communities are relatively rare and are part of the focus of this work. The present study was to locate and describe some of the current deciduous woodland communities in central Texas that contains *A. grandidentatum*. The most well-known site for viewing *A. grandidentatum* in Texas is the Lost Maples State Natural Area located just a few miles north of Vanderpool. This site has well documented *A. grandidentatum* populations and is a state property. However, not much is known about populations that may exist on private properties in central Texas.

PURPOSE

The primary objective of this project was to find and then gather baseline ecological information about *A. grandidentatum* and other low density deciduous woody plant populations in the Edwards

Plateau of central Texas. A secondary objective was to evaluate the potential use of satellite photography and unmanned aircraft to facilitate efficient identification and rapid assessment of deciduous woodlands that may have populations of *A. grandidentatum* and other low density woody species.

METHODS

The general area of interest in central Texas (Figure 1) is roughly bounded on the west by RM (ranch to market) road 187 and on the east by Texas State Highway 16. The north boundary is approximately RM road 39 and the south is bounded by RM road 476. This area is also commonly referred to as the "Swiss Alps of Texas". Study sites were established based on private property owner's willingness to allow access to their property. There were a total of five properties whose owners agreed to participate in the project.

Once confirmed, the properties were evaluated for deciduous woodlands using satellite imagery obtained in November 2016 during peak fall color change and freely available on Google Earth (Figure 2, example). Using the satellite imagery and conversations with the participating landowners, areas considered to be deciduous woodlands with a potential to have *A. grandidentatum* and other low density woody species were located (Figure 3). The possibility to have drone or un-manned aircraft (UA) flights were also established. Between November 13th and 25th, twenty-six drone flights were completed using a DJI Inspire 1 quadcopter flown at an altitude of 76.2 m (=250 ft AGL [above ground level] from the point of liftoff) in precise patterns across each area covering a total of 174 ha (430 acres). Upon completion 2,969 HD images were obtained to document areas thought to have deciduous woodlands and *A. grandidentatum* populations. Image resolution was approximately 1.0 inch per pixel.

Imagery was uploaded to Drone Deploy for stitching and then exported as a TIFF to ArcGIS 10 desktop software. Using the canopy color as a guide, the deciduous woodland communities were outlined then used to calculate the area of the deciduous woodlands using ArcGIS measurement tools. Areas were summed to get the total community area (ha). The images (JPGs) were used to determine where ground truthing should be done (Figure 4).

Next a matrix was established to rank the 26 areas based on the potential for *A. grandidentatum* presence and site accessibility. Ten were selected for ground-truthing and based on walk throughs, one of the 10 sites selected did not have any *A. grandidentatum* trees and another was inaccessible. This left 8 sites to be surveyed and all contained adult *A. grandidentatum* trees and all but one had *A. grandidentatum* juveniles. The ground surveys were conducted from April 6, 2018 through April 21, 2018. A total of 8900 m^2 (0.89 ha) in the eight sites were examined using the quadrat method (Van Auken et al. 2005). Although many more potential deciduous woodland communities were observed in the satellite imagery, the ability to fly the drone in these areas was limited because of FAA rules requiring visual line of sight at all times. One site was a north facing slope, and two sites were visually examined but no *A. grandidentatum* trees were found in one and the other was inaccessible. The communities were mostly located in deep, isolated, limestone canyons (Van Auken et al. 2017).

Domestic grazing was not occurring in the areas studied, but native herbivores were present. Elevation of the study area sites is approximately 480-620 m a. m. s. and soils were relatively deep calcareous silty clays (Mollisols over limestone bedrock, SCS 1979). Mean annual temperature is approximately 18.3°C, with a range from near 0.7°C in January to 34.1°C in August, and is highly variable. Mean annual precipitation is 72.4 cm/year with zero or very little in July and August and highly variable with May and September being wettest (World Climate 2011).

Density of all overstory trees found in these communities was determined in 5 m X 5 m or 25 m^2 quadrates (Van Auken et al. 2005). The number of 25 m^2 quadrats varied in each of the communities due

to site conditions and topography. Adequate sampling was demonstrated by examining species and density stabilization curves but is not presented. For overstory woody plants, there were a total of 356, 25 m^2 quadrats or 0.89 ha sampled in the deciduous communities. All plants greater than 137 cm in height and 3 cm basal diameter were considered trees and part of the overstory. They were identified (Correll and Johnston 1979; USDA-Plants 2019), counted and basal area was measured. Five 1 m^2 sub-quadrats were established in each of the 25 m^2 quadrats or 5780 m^2 to count understory woody plants (one in each corner and one near the center). All woody plants less than 137 cm in height and/or 3 cm basal diameter were identified and counted as seedlings or juveniles. Identity, density, relative density, basal area, and relative basal area were calculated for each overstory tree species and identity, density and relative density was determined for the understory woody plants within each community (Van Auken et al. 2005). Next, means were determined, but only species density and basal area and relative values are presented for overstory species. For the understory woody species, the juveniles, density and relative density are presented. Species richness (number of species) and % occurrence (%O = [# of communities species found in / total # of communities studied] x 100 is also presented.

RESULTS

General ecological characteristics of eight deciduous communities were identified using drone images and then examined with the quadrat procedure. The deciduous communities ranged from 0.29 ha to 4.45 ha in area with a total area of 9.56 ha (Table 1). Communities were mostly in the bottom of deep calcareous north-south canyons, with one a north facing hillside. Overstory species richness ranged from 5 to 14 species with 17 total overstory woody species found (Table 1). There were 11 to 21 woody species in the understory of each community with a total of 30 woody species present.

Total overstory density ranged from 153 to 1024 plants/ha with a mean of 559 plants/ha (Table 1). Mean overstory tree basal area was 25.47 m^2/ha and ranged from 9.01 to 34.81 m^2/ha (Table 1). *Juniperus ashei* and *Acer grandidentatum* had 100 % occurrence in the overstory, and were found in all eight communities studied (Table 2). *Quercus laceyi* and *Juglands microcarpa* were found in five communities for an occurrence of 63 %. *Quercus muehlenbergii*, *Diospyros texana*, *Fraxinus albicans* and *Q. buckleyi* had 50 % occurrence and were present in four communities. Nine other species had occurrences of < 50 % (Table 2). *Juniperus ashei* and *A. grandidentatum* had the highest mean overstory density at 221 and 169 plants/ha respectively with the other 15 species having densities between 1 and 41 plants/ha (Table 2). *Acer grandidentatum* had the largest mean basal area at 9.57 m^2/ha. *Quercus muehlenbergii*, *Q. laceyi* and *J. ashei* had the next highest basal areas at 6.10, 4.37 and 1.30 m^2/ha. The other 12 species had basal areas < 1.00 m^2/ha (Table 2).

There were 30 juvenile woody plants found in the understories of some of the eight communities examined. There were five tree species found in the understory of every community (100 % occurrence) including *Celtis laevigata*, *Q. buckleyi*, *J. ashei*, *D. texana* and *Prunus serotina* (Table 3). An additional five species (three tree species) were present in the understory of seven of the communities (87 % occurrence) including *Q. laceyi, A. grandidentatum, Fraxinus albicans* and two vines. The other 20 woody species in the understory had occurrences of 75 % or less (Table 3).

Total understory woody plant density ranged from 3710 plants/ha to 17025 plants/ha depending on the community, with a mean of 7963 plants/ha (Table 1). *Celtis laevigata* had the highest density followed by *Q. buckleyi, Q. laceyi, J. ashei, Q. muehlenbergii, A. grandidentatum* and *D. texana* in descending order (Table 3). All had mean density values that ranged from 1201 down to 602 plants/ha. The other 23 species had lower density values.

Acer grandidentatum juveniles were found in seven of the eight communities (87 % occurrence) with a mean density of 642 ± 700 plants /ha (Table 3). The other woody species juveniles were scattered

in various communities. In addition, the standard deviations for all of the species were relatively high. Almost all of the overstory trees had some seedlings or juveniles in the understory, but not all understory species had representatives in the overstory, for example. There were no *Celtis laevigata* trees.

DISCUSSION

The areas studied were steep, deep limestone canyons and one north facing hillside within the Edwards Plateau physiographic region of central Texas (Hill 1892; Bray 1904; Tharp 1939; Gould 1969; Correll and Johnston 1979; Amos and Gehlbach 1988). It is a physiographic region approximately 100,000 km^2 described by physical geology or geomorphology not one type of plant community. While it has been described simplistically as grasslands (Sims 1988) or Juniper woodlands (Amos and Gehlbach 1988), it is more accurately described as a diverse physiographic region that includes many plant communities that have a number of rare species and many endemic species (Pool et al. 2007; Van Auken 2018). Plant communities include several mixed juniper - oak woodlands as well as mesquite woodlands, shrublands, various types of savannas and grasslands in addition to riparian communities (Van Auken et al. 1979; Van Auken 1988; Diamond et al. 1995; Van Auken 2000; Van Auken and Ford 2017; Van Auken 2018).

Using satellite images and the drone ten deciduous woodland communities were identified. However, when examined on the ground one community did not have any *A. grandidentatum* plants and the second was inaccessible and neither were included in the present study. The current woodlands studied were similar to deciduous woodlands ecologically described over 40 years ago (Van Auken et al. 1981), and comparable to the communities reported in the upper canyons of this area (Palmer 1920). The current study was focused on *A. grandidentatum* populations within these deciduous woodland communities, but all of the woody species that were encountered were identified. Differences between the current study and the former study of these communities include 17 woody species (trees or shrubs) found in the current study while 19 were reported in the previous study (Van Auken et al. 1981). However, *Q. muehlenbergii, A. grandidentatum, Sideroxylon lanuginosum* and *Tilia caroliniana* were not found in the previous study. *Salvia ballotaeflora* (shrub) was reported in the previous study. Several species reported in the previous paper were only found in the understory of the current study.

Reasons for the large difference in woody plant density between the two studies (1851 vs. 8522 plants/ha, earlier study vs. current study) are probably in part location (the earlier study was more southern) and data collection times (late winter vs. late spring). In addition, in the earlier study, juveniles and mature plants were combined and reported as trees if their basal circumference was >3.0 cm, but if less they were not counted; but they were counted as juveniles in the current study. Also, *Aesculus pavia* was previously counted (per stem rather than per clump) thus having 296 ± 106 plants/ha versus 2 ± 4 plants/ha. *Diospyros texana* had a density ten times higher than the current studied, but why is uncertain. There was also a high density of *Q. laceyi* (= *Q. glaucoides*) and *Q. buckleyi* (= *Q. texana*) in these previously examined communities. The deciduous woodland communities in the current study were very open, with few understory shrubs. Understory density was high in most of the communities currently studied, but the woody plants were mostly less than 10 cm tall. In the understory of the current study there were 30 woody species including 16 juvenile tree species, 10 shrubs, and 4 woody vines whereas the understory was not reported in the previous study.

We were interested in the replacement dynamics of the species in the communities examined. However, little ecological or population information is available concerning the species present in these communities and communities dynamics was not part of the current study. Density values presented in the current document represent the mean number of woody plants found in the quadrates measured as overstory or understory plants/ha. We estimate the total area of the deciduous communities surveyed to be 9.56 ha. The mean density values presented in the current paper are per hectare and if the plants are

equally found through the deciduous communities examined, the actual number of plants of a given species would be expected to be 9.56 times higher because of the area of the deciduous woodlands. However, the communities seem to be structured with species, density and basal area changing with elevation and aspect, but exactly how they are structured is not known.

The relatively large number of *A. grandidentatum* juveniles found in the current study suggests that recruitment into the adult population could be occurring. Unfortunately, the fate of the juveniles in these communities is unknown. Mortality of all of them could occur annually or over many or all years. Recruitment could be episodic and only occur periodically with the interim between recruitment cohorts unknown. For long-lived species this is very difficult to know. In addition, there is a large degree of variability of the number of overstory trees and the number of understory individuals of the same species with little known of recruitment success for any of the species. Two previous studies showed little or no recruitment of *A. grandidentatum* juveniles (Nelson-Dickson and Van Auken 2016; Van Auken et al. 2017). However, more recently it has been reported that *A. grandidentatum* juvenile success is dramatically increased when juveniles are protected from large herbivores (Van Auken and Taylor 2020),

Although many more potential deciduous woodland communities were observed in the satellite imagery, these areas were not examined on the ground for various reasons. Mature or adult *A. grandidentatum* were present in some of the other areas, but density seemed to be low and plants were not readily observed on satellite photographs or with drone flights.

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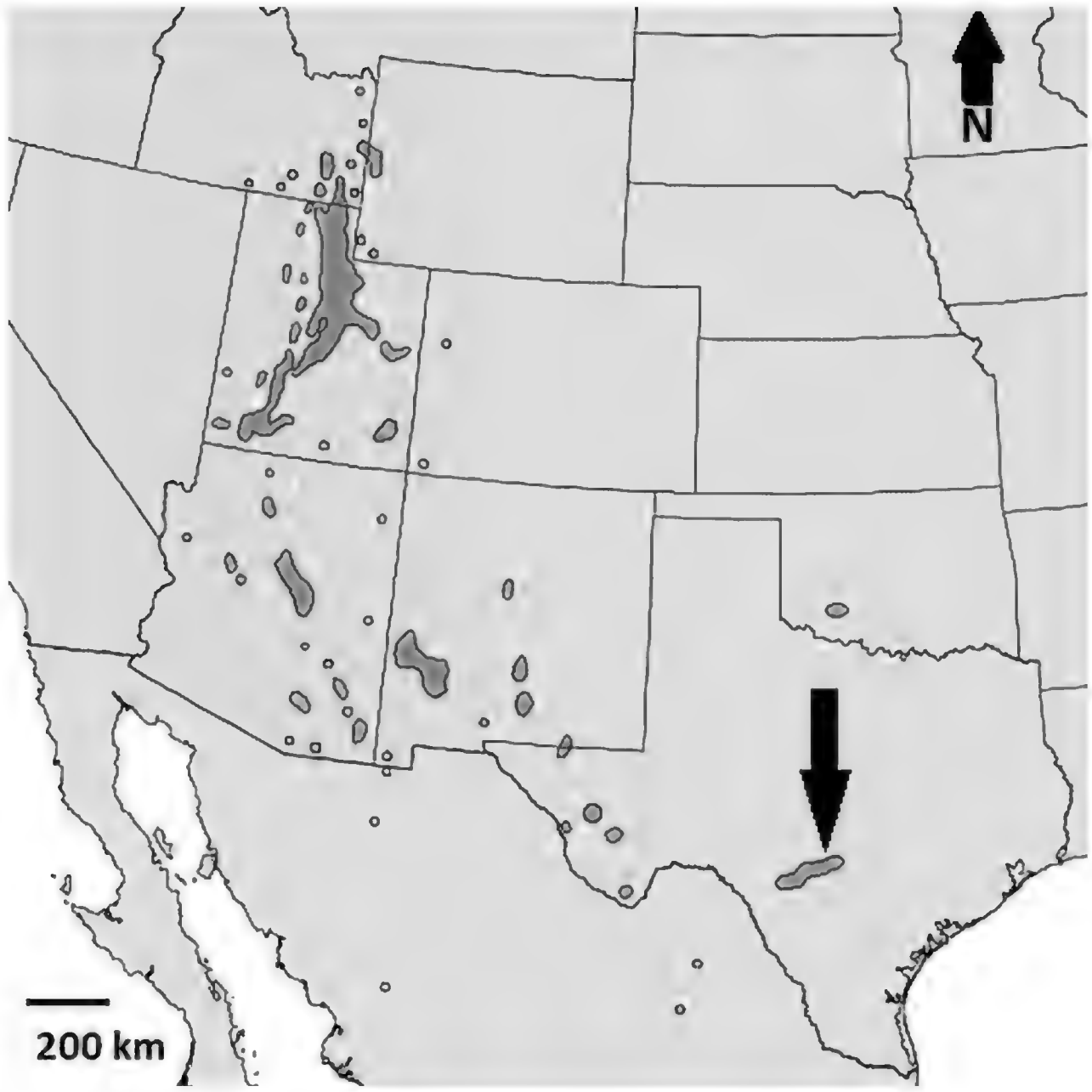

Figure 1. General locations of Bigtooth maple populations in central Texas and the western United States and Northern Mexico. The lower black arrow is the approximate location of the study sites of this project in central Texas.

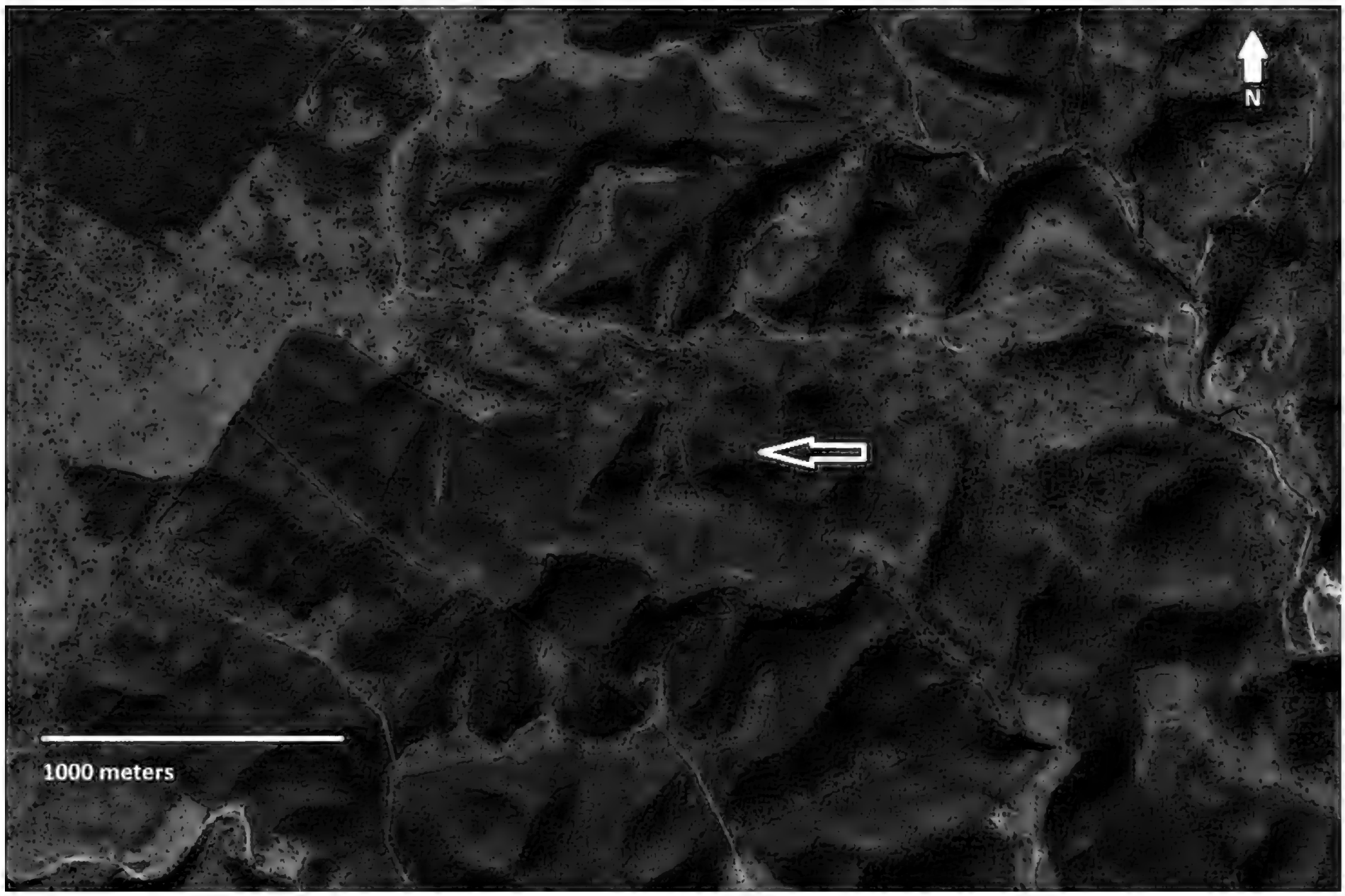

Figure 2. Example photograph from "Google Earth" of part of the study area taken in the fall of 2016 showing mostly the *Juniperus ashei* woodlands and various relatively deep limestone canyons where potential deciduous woodlands could occur. One of the deciduous communities is near the tip of the arrow but that part of the photograph has to be enlarged before the community can be easily seen.

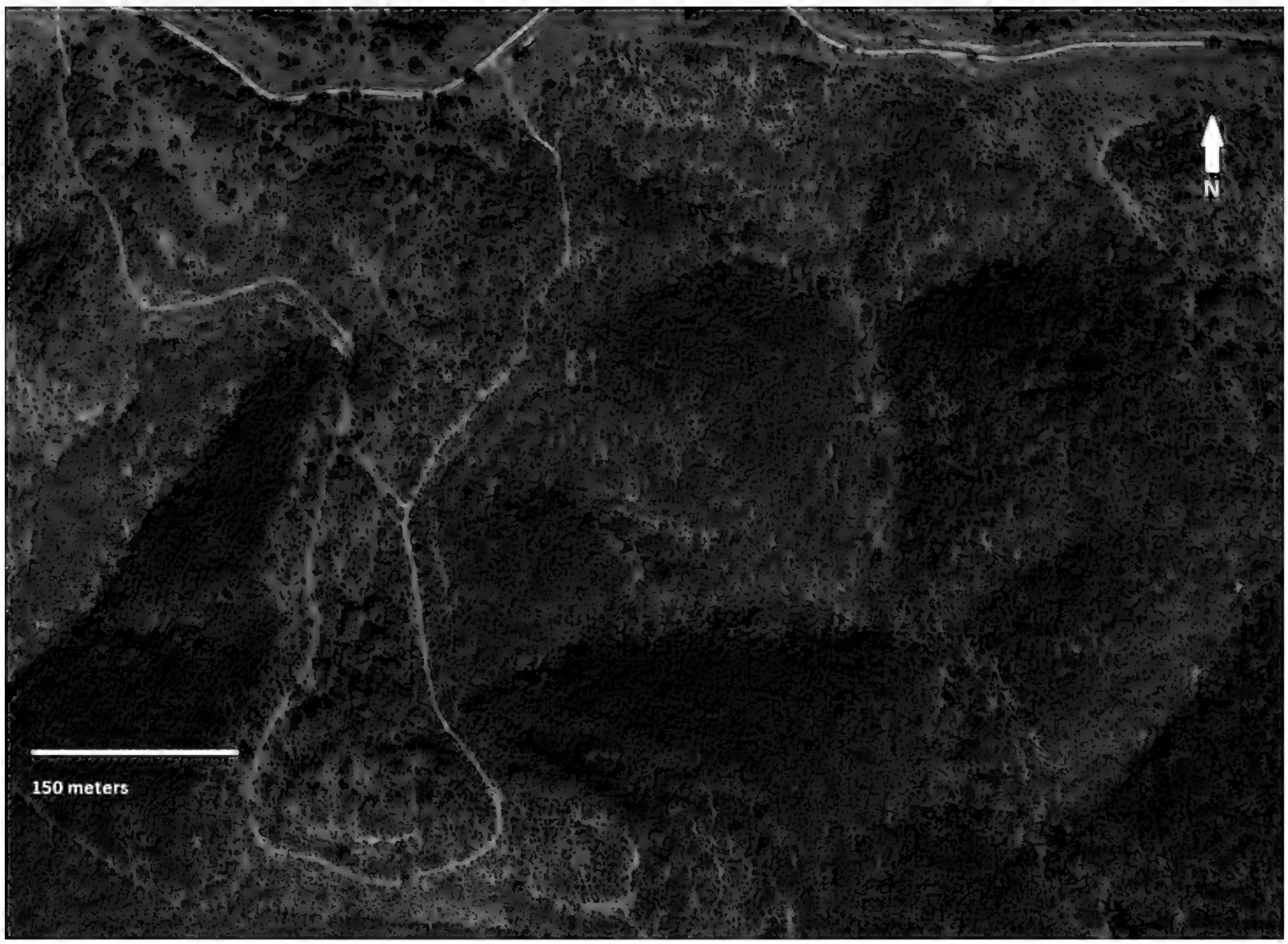

Figure 3. An example enlarged image of several potential deciduous woodlands with *Acer grandidentatum*. This picture was exported as a TIFF image and imported into ARCGIS-10.6 for assessment and characterizing the area of the deciduous woodland to determine if an *Acer grandidentatum* population was present. These deciduous woodlands can be easily seen because of the autumn colors of the leaves of some of the deciduous trees and include potential populations of *Acer grandidentatum.*

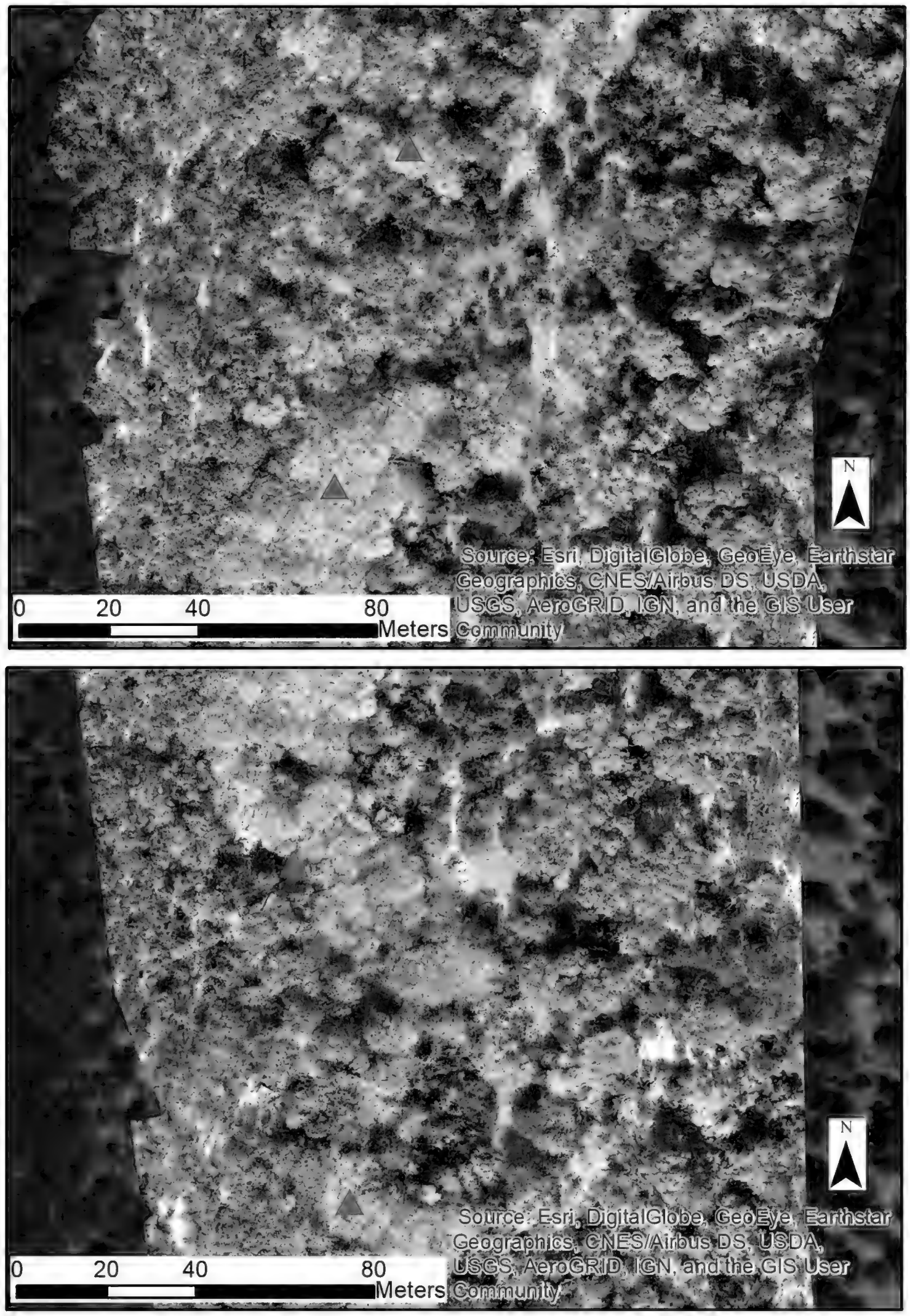

Figure 4. Example enlarged images of parts of two deciduous woodlands. These pictures were exported as TIFF images and imported into ARCGIS-10.6 for assessment and characterizing the area of the deciduous woodland to determine if *Acer grandidentatum* populations were present. These deciduous woodlands can be easily seen because of the autumn colors of the leaves of some of the deciduous trees and include potential populations of *Acer grandidentatum.* The large purple triangles indicate the approximate location of part of the ground quadrat transects.

Table 1. General measurements including the number of communities sampled, area of each community sampled, number of overstory and understory species as well as mean total overstory density and basal area and mean total understory density.

SAMPLE	AREA-ha	# OVER SPECIES	# UNDER SPECIES	DENSITY* OVER	DENSITY* UNDER	BASAL AREA**
1	0.96	5	15	153	5287	15.52
2	0.96	6	17	810	11895	28.96
3	4.45	5	21	183	17025	26.56
4	0.29	14	18	572	6021	23.42
5	0.29	9	15	645	5951	32.51
6	1.46	5	11	410	4372	32.97
7	0.79	7	12	675	3710	34.81
8	0.36	9	13	1024	9440	9.01
TOTAL or mean	9.56	17	30	559	7963	25.47

*plants/ha

**m^2/ha

Table 2. Summary of the overstory woody species found in the communities surveyed including all their percent occurrences, mean and relative densities in plants per hectare and mean and relative basal areas in meters squared per hectare.

SPECIES	% OCC*	MEAN** DENSITY	% DENSITY	MEAN*** BASAL AREA	% BASAL AREA
Juniperus ashei	100	221	40	1.30	5.20
Acer grandidentatum	100	169	30	9.57	38.26
Quercus laceyi	63	41	7	4.37	17.47
Quercus muehlenbergii	50	26	5	6.10	24.41
Diospyros texana	50	20	4	0.02	0.07
Sophora secundiflora	38	23	4	0.01	0.04
Vitis arizonica	38	14	3	0.03	0.11
Juglans microcarpa	63	10	2	0.44	1.75
Fraxinus albicans	50	9	2	0.93	3.71
Prunus serotina	25	6	1	0.67	2.67
Ungnadia speciosa	25	5	1	0.11	0.43
Quercus buckleyi	50	5	1	0.28	1.11
Sideroxylon lanuginosum	25	5	1	<0.01	< 0.01
Juglans major	13	1	0	0.44	1.77
Celtis laevigata	13	1	0	0.06	0.25
Tilia caroliniana	13	1	0	0.24	0.97
Aesculus pavia	13	2	0	<0.01	<0.01
TOTAL		559	100	25	98.18

*OCCURRENCE

**PLANTS/ha

***m^2/ha

Table 3. Summary of the understory woody species found in the communities surveyed including all their percent occurrences, mean and relative densities in plants per hectare.

UNDERSTORY SPECIES	% OCCURRENCE	MEAN DENSITY*	SD	% DENSITY
Celtis laevigata	100	1201	2450	15
Quercus buckleyi	100	1059	430	13
Quercus laceyi	87	926	941	12
Juniperus ashei	100	667	753	8
Quercus muehlenbergii	75	658	704	8
Acer grandidentatum	87	642	700	8
Diospyros texana	100	602	445	8
Parthenocissus quinquefolia	87	386	545	5
Sideroxylon lanuginosum	63	273	519	3
Vitis arizonica	75	257	307	3
Smilax bona-nox	87	249	234	3
Sophora secundiflora	50	241	370	3
Ungnadia speciosa	50	204	412	3
Fraxinus albicans	87	158	188	2
Prunus serotina	100	138	106	2
Ilex decidua	13	99	279	1
Juglans microcarpa	63	46	70	1
Ulmus crassifolia	13	37	104	<1
Mahonia trifoliolata	25	26	57	<1
Rhamnus caroliniana	25	24	50	<1
Toxicodendron radicans	25	12	30	<1
Ptelea trifoliata	13	11	31	<1
Tilia caroliniana	13	10	28	<1
Styphnolobium affine	13	7	19	<1
Ageratina havanensis	13	7	19	<1
Cercis canadensis	25	6	11	<1
Yucca rupicola	13	6	16	<1
Baccharis neglecta	13	6	16	<1
Rhus virens	13	4	10	<1
Styrax platanifolius	13	4	10	<1
Juglans major	13	<4	10	<1
Aesculus pavia	13	<4	10	<1
Eysenhardtia texana	13	<4	10	<1
Quercus fusiformis	13	<4	10	<1
Platanus occidentalis	13	<4	10	<1
Garrya ovata	13	<4	10	<1
TOTAL		7963		98

*PLANTS/ha

Putative late Pleistocene hybrids inferred from volatile leaf oils (terpenoids) of *Cupressus chengiana*

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ABSTRACT

An investigation of variation in essential leaf oils of *Cupressus chengiana* from three populations (BLJ, Gansu; DDH, Sichuan, MJR, Sichuan) found the oils were high to moderate amounts of sabinene, α-pinene, myrcene, limonene, β-phellandrene, γ-terpinene, umbellulone, terpinen-4-ol, δ-cadinene, germacrene-D, elemol, hedycaryol, iso-abienol, and trans-totarol. A Minimum Spanning Network based on 15 major terpenoids revealed the 34 oils grouped by population, except 5 trees from DDH had oils more like BLJ plants. PCO ordination showed the 5 unusual DDH oils grouped in a position suggesting they are hybrids or of hybrid origin. The ordination of one plant suggested it may be a backcross to BLJ plants. DNA sequencing has inferred that DDH and BLJ ancestors hybridized during the Quaternary (Li et al. 2020), but the presence of chemical intermediate oils in DDH, seems to imply that more recently (late Pleistocene), a second hybridization event occurred between DDH and BLJ. Correlation among the 15 major compounds revealed high correlation between structurally similar compounds. The potential use of the presence of several chemical-types to analyze biochemical pathways is discussed. *Published on-line www.phytologia.org Phytologia 1022(2): 41-54 (June 24, 2020). ISSN 030319430.*

KEY WORDS: *Cupressus chengiana*, leaf terpenoids, essential oils, composition variation, sabinene, α-pinene, umbellulone, elemol, eudesmols, trans-totarol.

Cupressus chengiana S. Y. Hu, the Minjiang cypress, grows in the eastern Qinghai-Tibet Plateau (QTP), mostly in arid valleys at the headwaters of three rivers: Sichuan: Minjiang and Daduhe rivers, and Gansu: Bailongjiang river (Fig. 1), at 800 to 2900 m (Xu et al. 2017; Li et al. 2020). The Minjiang cypress has experienced a large decline in population sizes due to logging and grazing (Hao et al. 2006) and, as such, has been listed as 'Second-class Endangered Plant' in the *Red Book of China: Rare and Endangered Plants* (Fu, 1992). Xu et al. (2017) found evidence of reduced gene flow between the Gansu and Sichuan populations.

Li et al. (2020) studied *C. chengiana* sampled from DDH, MLR populations in Sichuan and BLJ populations in Gansu (Fig. 1). Using High-throughput Sequencing (HTS), they utilized 31,527 nuclear SNPs for phylogenetic analysis of the three groups. A ML tree based on these nuclear data (Fig. 2) shows that the three taxa (or ESU, Evolutionary Significant Units) are in three highly supported, distinct clades: DDH, MRJ and BLJ.

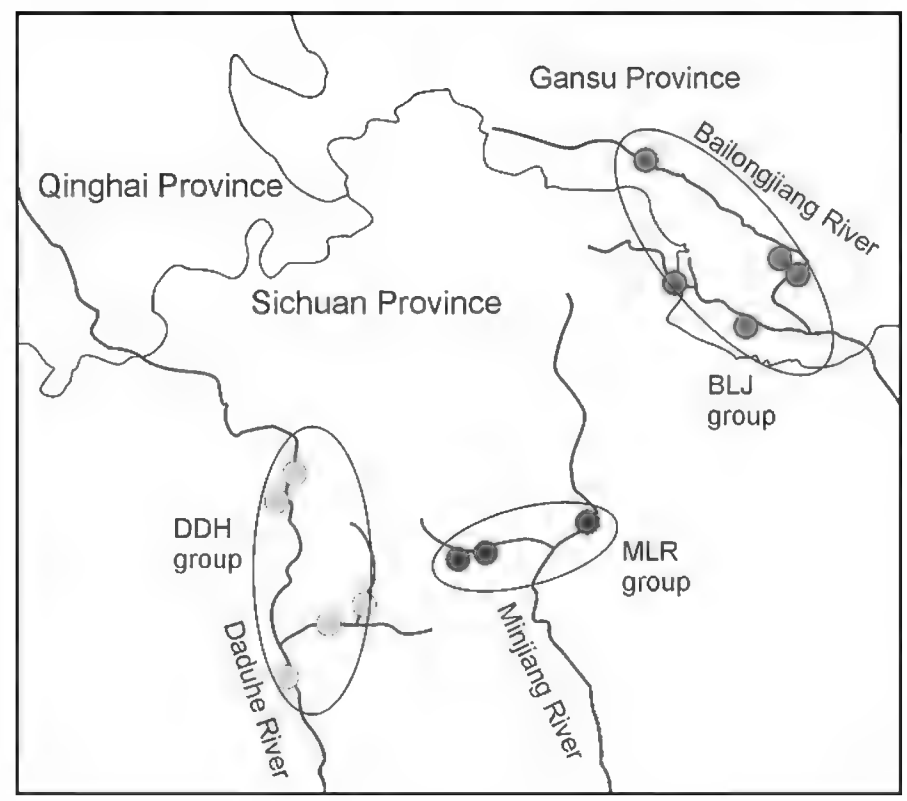

Figure. 1. Distribution of *C. chengiana* populations sampled (adapted from Li et al. 2020).

MJR group, Minjiang River, Sichuan grouped with the BLJ group, Gansu, but appeared intermediate in a PCA ordination (Li et al. 2020). In fact, Li et al. (2020) also investigated cp (chloroplast) ML tree and found that DDH and MJR apparently share the same cp haplotype lineage, as the DDH and MJR samples were all intermixed in a distinct clade in the ML tree, and grouped in highly supported clades as ((DDH, MJR), (BLJ). This is excellent evidence of chloroplast capture (see Adams, Schwarzbach and Tashev, 2016; Adams 2016; Farhat et al. 2019; Hojjati et al. 2019; Adams et al. 2017). Thus, *C. chengiana*, MJR group, on the Minjiang River, appears to be of hybrid origin between male DDH and female BLJ in the Quaternary when the groups descended to lower, warmer, dryer, elevations to produce areas of sympatry (Li et. al. 2020).

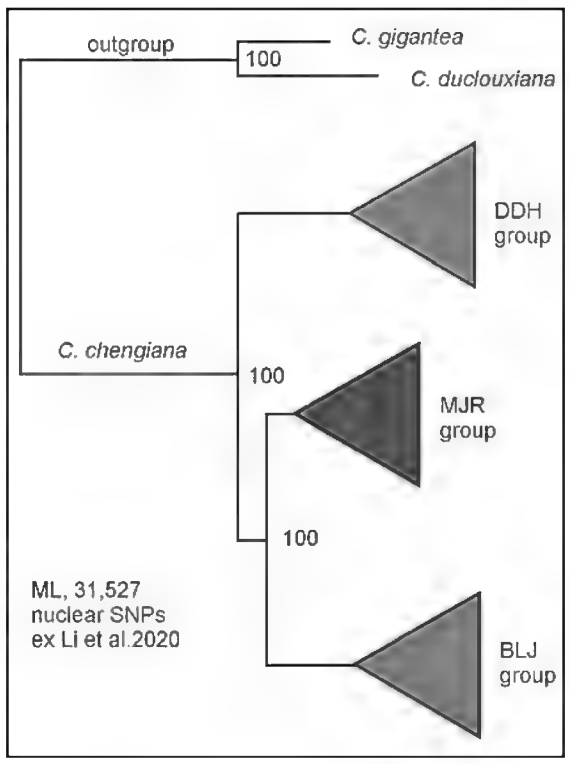

Fig. 2. ML tree showing the grouping of MJR and BLJ. Based on data in Li et al. 2020.

The DDH, MJR and BLJ ESUs (taxa) are, morphologically, difficult to distinguish. With the collection of extra foliage in the Xu et al (2017) field study, this presented us with an unusual opportunity to examine the volatile leaf oil of *C. chengiana* from the three DDH, MJR and BLJ taxa.

The volatile leaf oil of *C. chengiana* has not been extensively analyzed. The most detailed report on the composition (Cool et al. 1998) was from oil obtained from a natural population (10 samples) at Wu Du, Gansu (Kansu), 33˚ 34' N, 104˚ 55' E, 1500m. This corresponds to the BLJ population group, site Wudu (Table 1, Xu et al. 2017). Cool et al. (1988) reported the oil was dominated by sabinene (24.9%), elemol/hedycaryol (14.5%), trans-totarol (11.7%), with moderate amounts of α-pinene (5.8%), β-pinene (2.8%), limonene (2,2%), germacrene-D (2.5%), iso-abienol (3.1%), and sempervirol (2.4%).

Li et al. (2005) analyzed the leaf essential oils from three natural populations on the Minjiang and Daduhe rivers and one man-made forest in Wenchuan. The oil from the artificial forest was quite different from the oils the three natural populations. The concentrations of the larger components of the oil from the three natural populations were similar. Analysis of the leaf oil of *C. chengiana* cultivated in France (Pierre-Leandri et al. 2003) reported that α-pinene (17.6%) and sabinene (32.1%) were the major components, with moderate amounts of myrcene (3.6), α-terpinene (2.5), limonene (+β-phellandrene?) (5.3), terpinolene (2.4), terpinen-4-ol (11.3) and elemol (2.9%). Unfortunately, the origin of their *C. chengiana* was only given as 'China', so we cannot know if he analyzed DDH, MRJ or BLJ plant(s).

The purpose of this paper is to present a detailed analysis of the oil compositions DDH, MRJ or BLJ plants as collected and examined by Xu et al. 2017 and Li et al. 2020.

MATERIALS AND METHODS

Plant collection of *C. chengiana* by locations:
Population DDH *Lab acc. Robert P. Adams 15732-15743*(12): 31˚ 01' 44.93" N 102° 15' 0.93" E., 2252.8 - 2711.7 m, Aug 2019, Sichuan, China Coll. Kangshan Mao. ns. LXT-05;

Population MJR *Lab acc, Robert P. Adams 15744-15753*(10): 31° 38' 23.52" N 103° 48' 20.97" E., 1742.68- 2073.3 m, Aug 2019, Sichuan, China, Coll. Kangshan Mao, ns. LXT-16;

Population BLJ: *Lab acc. Robert P. Adams 15754-15765(12)*: 33° 15' 11.82" N 104° 59' 01.44" E., 1742.68- 2073.3 m, Aug 2019, Gansu China
Coll. Kangshan Mao, ns. ZR-20;

Isolation of Oils - Fresh leaves (200 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

Volatile oil Analyses - Oils from 10-12 trees of each of the taxa were analyzed and average values are reported. The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams 2006 for operating details). Identifications were made by library searches of the Adams volatile oil library (www.juniperus.org, Adams, 2006), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

Data Analysis - Terpenoids (as per cent total oil) were coded and compared among the species by the Gower metric (1971). Similarities between oils were computed as formulated by Adams (1975). PCO (Principal Coordinate analysis) was performed by factoring the similarity matrix using the formulation of Gower (1966) and Veldman (1967). PCA (Principal Component Analysis) done on the raw % concentration data for 15 terpenoids for each of the 34 individuals using a Fortran program (RPA) based on the formulation in Veldman (1967).

RESULTS

The composition of the leaf essential oils of *C. chengiana* for populations DDH, MJR, and BLJ, were found to be quite variable with chemotypes (cpds. being absent/present among samples). To explore the variation in oil composition, 15 of the terpenoids of largest concentration were coded and analyzed by a minimum spanning network (MSN) and this revealed the three taxa (ESUs), BLJ, DDH and MJR are mostly distinct (Fig. 3). BLJ oils formed a very tight cluster for samples B2, B4-B6, B8-B10. However, three oils, B1, B3, and B7, were quite different from the typical BLJ oils (Fig. 3). Notice that B1 and B3 are not very similar to any of the oils (Fig. 3).

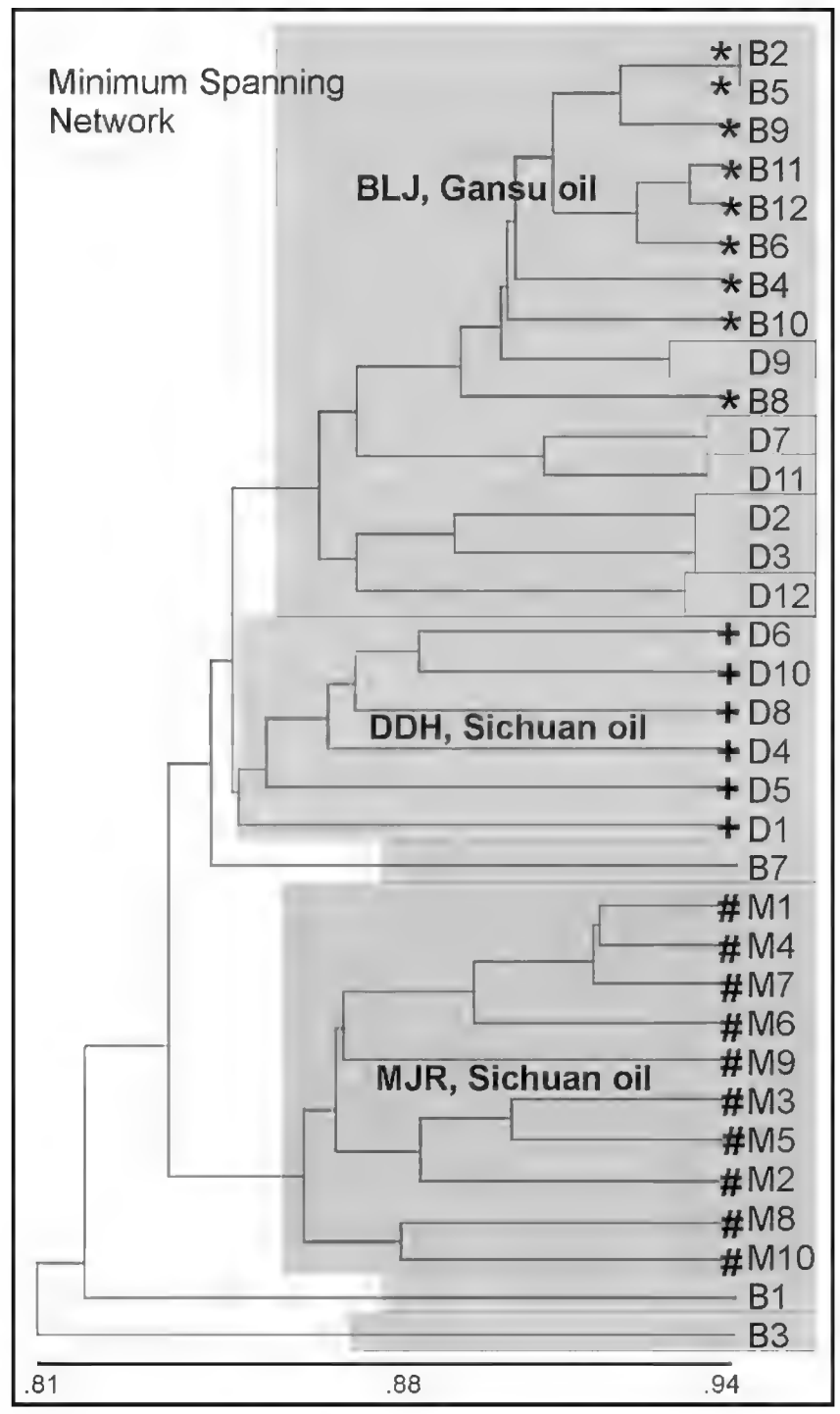

Figure 3. MSN (Minimum Spanning Network) based on 15 cpds. Individuals with *, +, # were used in the Avg. oils for DDH, MJR and BLJ.

Six of the DDH oils (D9, D7, D11, D2, D3, and D12 were most similar to the BLJ oils (Fig. 3). This might indicate that these plants are of hybrid origin as the terpenoids are inherited as intermediate concentration, dominant/ recessive and/ or transgressive (larger or smaller than the compound in either parent) (*Cryptomeria*, Adams and Tsumura 2012; *Pseudotsuga*, Adams and Stoehr 2013).

Table 1 reveals that only a few compounds distinguish between all three MRJ, DDH and BLJ oils (Table 1). The most predictive compound is sabinene: MRJ, low: 6.0%; DDH, medium: 11.3%, BLJ, high:

24.5%). The MJR oil is lower in sabinene and terpen-4-ol. DDH and MJR (ex Sichuan) are high in α-pinene and umbellulone and low in elemol/hedycaryol, α- and β-eudesmol, and α-cadinol. DDH and BLJ are high in sabinene, terpinen-4-ol, and low in cis-murrola-4(14),5-diene and epi-zonarene. Finally, MJR and BLJ are low in α-cadinol.

Table 1 also includes information from Cool et al. (1998). Generally, the three populations oils are very similar. Only one compound (linaly acetate) is absent in DDH and MJR, and a trace in BLJ. However, the absence of trace components is equivocal, as an increased injection of oil can generate sufficient ions in mass spectroscopy to detect that the compound as 'present'.

BLJ and Cool (both ex Gansu) are both high in sabinene and elemol/hedycaryol and lower in α-pinene, umbellulone, cis-murrola-4(14),5-diene, and epi-zonarene. In general, the Gansu oils are very similar (BLJ and Cool, Table 1). Three unknown diterpenoid compounds were encountered in the oils of DDH, MJR, and BLJ, all with a molecular weight of 316. Interestingly, 2 of these diterpenoids (KI 2341 and 2364) were also reported as unknowns by Cool et al. (1998). Searches of NIST MS database revealed no similar compounds. Isolation and NMR will be needed to identify these compounds.

A detailed analyses (Table 1) of volatile leaf oil compositions with the 2 (D7, D9, D11 not shown due to space) of the 6 DDH oils (D9, D7, D11, D2, D3, and D12), reveal that these oils are similar to BLJ Avg., in several compounds: α-thujene (1.1 - 1.3%), α-pinene (4.5 - 6.4), sabinene (28.8 - 35.7), α-terpinene (1.8 - 1.3), β-phellandrene (1.4 - 1.7%), γ-terpinene (2.3 - 2.9), cis-sabinene hydrate (0.8 - 1.2), terpinolene (1.3 - 1.6), cis-p-menth-2-en-1-ol (0.4 - 0.6), camphene hydrate (t, trace in all 3), terpinen-4-ol (5.1 - 6.6), trans-piperitol (0.2 - 0.3), bornyl acetate (t - 0.2), α-cadinol (0.4 - 1.7), abietatriene (0.2 - 0.6), iso-abienol (1.6 - 3.1) and cis-totarol, methyl ether (t - 0.3). So, it is easy to see why D7 and D9 (and D11, D2, D3, D12) cluster with BLJ.

The oils of the unusual oils of B3 and B7 are included in Table 1 (B1 oil is also unusual but not included due to space). B3 oil is very low in sabinene (only 1%) and high (14.1%) in elemol/ hedycaryol (combined as they elute together and have the same MS pattern); β-eudesmol (9.0%), and α-eudesmol (7.8%). B3 is especially high in trans-totarol (16.8%). B7 oil is similar to B3, but both are very different from typical BLJ oil (Table 3).

To better visualize the relationships among the individuals, PCO was run using the 15 terpenoid set to construct a similarity matrix. Factoring the similarity matrix resulted in 7 eigenroots larger than the average matrix diagonal value. The eigenroots asymptoted after 6 roots and these accounted for only 66.84% of the variance among the 34 individuals. The presence of chemotypes among the plant oils likely creates mini-groupings that impede the extraction of variance with fewer eigenroots. The first three eigenroots accounted for 53.66% of the variance and were used for 3D PCO ordination (Fig. 4).

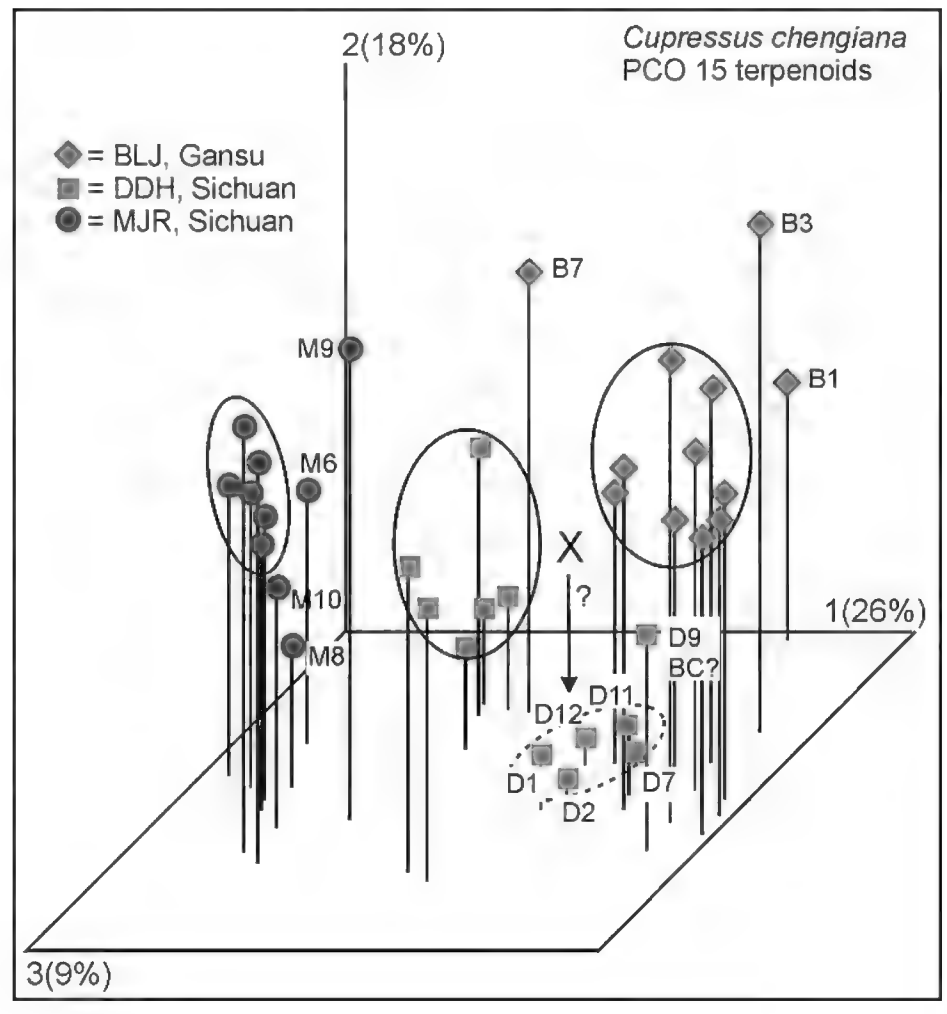

Figure 4. PCO ordination. The solid-line ellipses are the oils used to construct the averages for BLJ, DDH, and MRJ in Table 1.

The PCO ordination distinguishes the three groups (DDH, MJR, BLJ) shown by MSN (Fig. 3). MJR appears split with 4 (M6, M8, M9, M10) grouping somewhat with DDH. Oils within the BLJ solid-line ellipse are those with an * in Figure 3, and were used for the average oil in Table 1. DDH is clearly divided into the 'typical' group (+ sign in MSN, Fig. 3) and the 'hybrid' group (dashed-line ellipse) near the base of the BLJ group. Notice that the 'typical' DDH, 'typical' BLJ and the DDH 'hybrids' (dashed-line ellipse) form a 'V' or 'U'. The 'V' shape with the parents at the upper corners and the hybrids at the base of the V has been shown to be characteristic of both artificially obtained hybrids in fish and natural hybrids in *Juniperus* (Adams 1982). It has also been verified in artificial hybrids in *Cryptomeria* (Adams and Tsumura 2012) and *Pseudotsuga* (Adams and Stoehr 2013). The 'hybrids' thus appear to be from DDH x BLJ (Fig. 4). Notice that D9 is intermediate between the 'hybrids' and BLJ, and might be a backcross. The hybrid origin of MJR in Quaternary when the taxa were forced southeast of their present range into a refugium that was warmer and drier (Li et al. 2020) seems plausible. However, the, presumedly, more recent crosses of DDH x BLJ (Fig. 4), favor a second migration of DDH and BLJ in order for the taxa to be in breeding proximity. This latter hybridization event could have been recent, during the last glacial advance and retreat (ca. 12-14,000 ybp, the Late Deglaciation) (Cheng et al., 2018) with range expansion into the DDH and BLJ present ranges. One should note that it is possible that the divergent oils in the 'hybrids' may be nothing more than convergence by random drift, leading to oil profiles similar to BLJ. Additional research is needed to clarify the situation.

Correlation among terpenoids:

PCA (Principal Component Analysis) was performed to examine the correlation between the 15 major compounds (Table 2). Eigenroots from the correlation matrix asymptoted after 5 eigenroots and these accounted for 88.53% of the variance among the 15 terpenoids. The first 3 principal components accounted for 37.6, 28.4 and 13.6% of the variance among the 15 terpenoids. Ordination (Fig. 5) shows the terpenes (C10 hydrocarbons) are from two pathways: sabinene- γ-terpinene-terpinen-4-ol; and α-pinene-terpinolene-myrcene. The terpene alcohol, terpinen-4-ol, groups with the C10-HC (Fig. 5). Umbellulone (UMBO, C10-ketone) is loosely associated with the APNN-TRPN-MYRC group.

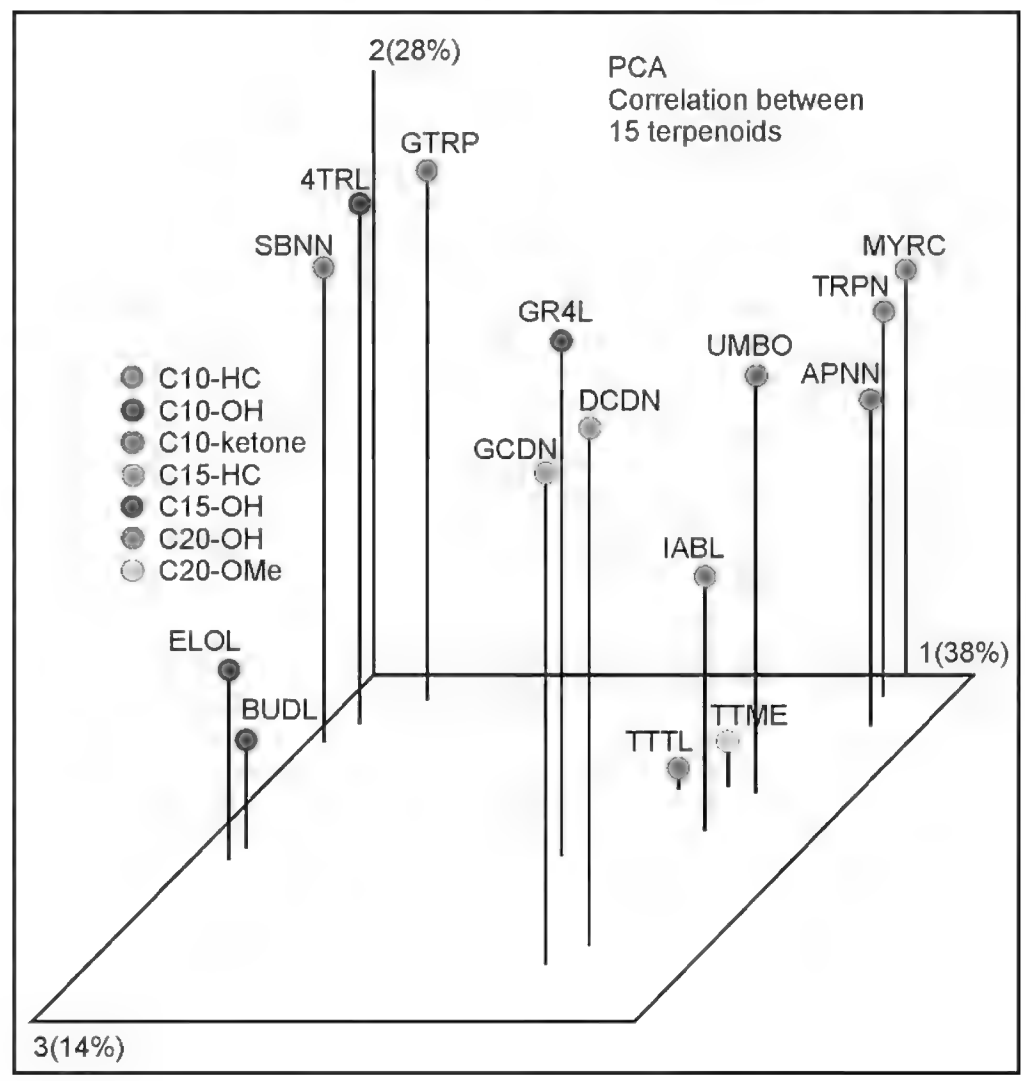

Figure 5. PCA ordination of 15 terpenoids.

Two sesquiterpenes, δ- and γ-cadinene, are highly correlated and also correlated with germacrene D-4-ol, a sesquiterpene alcohol. The other two sesquiterpene alcohols (elemol, β-eudesmol) are highly correlated but quite separated from germacrene D-4-ol (Fig. 5). No significant amounts of diterpene hydrocarbons were present, but oxygenated diterpenes were present in the oils. Two structurally related diterpenes, trans-totarol and trans-totarol, methyl ether, are highly correlated (0.72) and group together. Another diterpene alcohol, iso-abienol, groups loosely with trans-totarol and trans-totarol, methyl ether (Fig. 5).

Examining the populations with the 15 terpenoids, arranged in correlated groups (Table 3), clearly shows the 6 'hybrid' oils are high in sabinene as is common in the BLJ population. This is more clearly

seen in Table 4, where plant oils are sorted by sabinene. Notice the highest (gold highlight) concentrations of sabinene are in 2 DDH 'hybrids' (D8, 35.7%; D11 33.2%), followed closely by 3 BLJ plants, then 4 more DDH 'hybrids' (Table 4). B3, an unusual BLJ plant, has very low sabinene. NextGen analyses comparing B3 (and also M9) exomes with D9, D11, B9, D2, B5 might prove useful in discovering differences in sabinene synthetase genes.

There are 2 chemotypes of elemol: very high and trace amounts (Table 5). NextGen analyses comparing B1 (another very unusual oil type) exome with D1, D12, D2, D3, D5, (as well as M2, M3, M5) exomes should be useful in examining differences in elemol synthetase genes.

Umbellulone is an unusual terpene ketone that is common in *Cupressus* (Cool et al. 1998). Although it is not extremely in high concentration (8.8%, D8), it is very frequently only a trace (0.05% or less) in 9 plant oils (Table 6). Again, NextGen analyses comparing the D8 exome with the exomes of D9, D11, etc., Table 6) could uncover differences in umbellulone synthetase gene(s).

Finally, germacrene D-4-ol also shows an interesting pattern (Table 7) in that the highest concentration is in one of the 'hybrids', D12 (5.8%) and the lowest concentration is in another 'hybrid' D9 (0.02%). Contrasting their exomes should prove very informative. It is significant that for the aforementioned chemotypes (sabinene, elemol, umbellulone, germacrene D-4-ol), the largest concentration was found in either D8, D9, D12 or B1. The 'hybrids' and atypical (B1) plants express transgressive variation that is a very common feature of the oils of hybrid conifers (*Cryptomeria*, Adams and Tsumura 2012; *Pseudotsuga*, Adams and Stoehr 2013).

In summary, the unusual chemical variation between and especially within BLJ and DDH populations make these resources a valuable biological resource that should be protected.

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Table 1. Compositions of the leaf oils of *Cupressus chengiana* from three populations: DDH Avg. oil (15876) = 15732,35,36,37,39,15743(6). MRJ Avg. oil (15829) = 15744-15753(10). BLJ Avg. oil (15879) = 14655, 57,58,59, 15761-65(9). Individuals D7(15738) and D9(15740) grouped with BLJ oil by MSN (Fig. 3) and B3(15756) and b7(15760) were unusual oils that are not similar to any oils (MSN, Fig. x). Shading: High values (yellow), medium (blue), low (green). Gold is very unusual high conc. and turquoise is very unusual low conc.

		Avg.	Avg.	oils like BLJ		Avg.	unusual BLJ oils		
KI	compound	MJR, Sichuan 15829	DDH, Sichuan 15876	DDH-D7 15738	DDH-D9 15740	BLJ, Gansu 15879	BLJ-B3 15756	BLJ-B7 15760	Cool et al.[1] Gansu
921	tricyclene	t	t	t	t	t	t	t	
924	α-thujene	0.6	0.6	1.3	1.1	1.1	0.1	0.5	0.9
932	α-pinene	18.5	14.1	6.4	4.5	6.3	3.7	5.5	5.8
946	camphene	0.3	t	t	t	t	t	0.1	
969	sabinene	6.0	11.3	28.8	35.7	24.5	1.0	9.3	24.9
974	β-pinene	0.6	0.4	0.2	t	0.2	0.2	0.2	0.1
988	myrcene	3.1	2.6	2.3	2.2	2.3	0.6	1.4	2.8
1002	α-phellandrene	0.1	0.1	0.1	t	t	t	t	
1008	δ-3-carene	t	t	-	-	t	t	t	
1014	α-terpinene	0.8	1.0	1.8	1.5	1.3	0.1	0.5	0.2
1020	p-cymene	0.2	0.2	0.5	0.2	0.2	t	0.1	0.1
1024	limonene	3.4	1.2	1.1	0.6	1.4	0.9	0.8	2.2
1025	β-phellandrene	3.4	2.7	1.7	0.8	1.4	0.6	0.8	1.0
1044	(E)-β-ocimene	0.2	t	0.1	t	t	t	t	
1054	γ-terpinene	1.3	1.6	2.9	2.5	2.3	0.3	0.9	0.3
1065	cis-sabinene hydrate	0.2	0.4	1.2	1.2	0.8	0.1	0.4	0.4
1086	terpinolene	2.7	1.9	1.6	1.3	1.6	0.5	0.8	1.1
1097	trans-sabinene hydrate	0.6	0.4	0.7	0.7	1.0	0.5	0.9	0.1
1097	linalool	0.6	0.5	0.7	0.7	0.9	0.5	1.1	1.3
1117	4-methoxy thujone	0.1	0.1	0.2	0.2	0.4	t	0.2	
1118	cis-p-menth-2-en-1-ol	0.1	0.3	0.6	0.5	0.4	t	0.2	
1122	α-campholenal	t	t	t	t	t	t	t	
1136	trans-p-menth-2-en-1-ol	0.2	0.2	0.4	0.3	0.2	0.1	0.1	
1145	camphene hydrate	0.3	0.3	t	t	t	0.3	0.4	
154	karahanaenone	t	t	t	t	0.1	0.1	0.1	0.2
1165	borneol	0.2	t	t	t	t	t	t	
1167	umbellulone	3.2	4.1	3.2	t	0.8	t	t	0.3
1174	terpinen-4-ol	2.1	3.8	6.6	6.2	5.1	1.0	2.0	0.5
1189	p-cymen-8-ol	t	t	0.2	t	t	t	t	
1186	α-terpineol	0.3	0.4	0.4	0.3	0.4	0.2	0.1	t
1195	cis-piperitol	t	t	0.2	0.2	t	t	t	
1207	trans-piperitol	t	0.1	0.3	0.2	0.2	t	t	
1232	thymol, methyl ether	t	t	t	-	t	t	t	
1241	carvacrol, methyl ether	t	t	t	-	t	t	t	
1249	piperitone	t	t	0.1	t	t	t	t	
1254	linalyl acetate	-	-	-	-	t	t	t	0.2
1287	bornyl acetate	0.5	0.5	t	t	0.2	0.7	0.6	0.1
1298	carvacrol	t	t	t	t	t	t	t	
1345	α-terpinyl acetate	1.0	0.5	0.7	0.3	1.1	1.0	1.1	0.9
1417	(E)-caryophyllene	0.8	0.3	0.3	0.2	0.2	0.3	0.2	0.5
1429	cis-thujopsene	t	0.4	1.4	0.2	1.0	1.9	1.1	1.2
1435	cis-muurola-3,5-diene	1.9	t	t	3.8	0.6	t	1.9	0.6
1452	α-humulene	0.4	t	t	t	0.2	0.4	0.2	0.2
1465	cis-muurola-4(14),5-diene	4.6	0.3	0.2	9.8	1.5	0.2	4.5	1.8
1478	γ-muurolene	0.2	0.5	0.5	t	0.2	0.3	0.2	
1480	germacrene D	2.0	0.7	0.5	0.6	0.6	0.6	1.4	2.5
1493	trans-muurola-4(14), 5-diene	0.2	0.3	0.2	t	t	0.2	t	
1501	epi-zonarene	1.1	t	t	t	0.3	t	t	0.2
1500	α-muurolene	0.3	1.2	0.9	2.2	0.3	0.7	0.9	

		Avg.	Avg.	oils like BLJ		Avg.	unusual BLJ oils		
KI	compound	MJR, Sichuan 15829	DDH, Sichuan 15876	DDH-D7 15738	DDH-D9 15740	BLJ, Gansu 15879	BLJ-B3 15756	BLJ-B7 15760	Cool et al.1998 Gansu
1513	γ-cadinene	0.7	1.7	1.3	t	0.4	0.6	0.6	t
1522	δ-cadinene	2.0	4.4	3.3	0.7	1.2	1.8	0.6	t
1533	10-epi-cubebol	0.3	0.2	t	t	0.2	t	t	0.4
1533	trans-cadina-1,4-diene	t	t	t	t	t	t	t	
1537	α-cadinene	t	0.4	0.3	0.9	t	t	t	
1549	elemol + hedycaryol (1:6)	1.8	3.0	4.8	4.3	11.5	14.1	14.8	14.5
1559	cis-muurola-5-en-4-α-ol	0.3	t	t	1.7	0.2	t	0.7	0.5
1574	germacrene D-4-ol	0.5	2.9	4.3	t	0.8	1.1	t	0.2
1600	cedrol	0.2	0.1	0.4	t	0.3	1.1	0.5	0.2
1607	β-oplopenone	t	0.2	0.4	0.3	t	0.8	0.4	
1618	(1,10)-di-epi-cubenol	0.2	t	t	t	t	t	t	
1630	γ-eudesmol	0.3	0.6	1.2	0.8	1.8	4.3	2.7	
1638	epi-α-cadinol	0.6	1.8	1.1	0.3	0.5	1.2	0.4	
1638	epi-α-muurolol	0.6	1.9	1.2	0.3	0.5	1.3	0.5	
1644	α-muurolol	0.2	0.7	0.4	t	t	t	t	
1649	β-eudesmol	0.5	1.2	2.0	1.4	3.8	9.0	5.4	0.3
1652	α-eudesmol	0.6	1.6	2.1	1.2	3.2	7.8	3.6	0.6
1653	α-cadinol	1.6	3.5	1.7	0.8	0.4	t	-	
1675	cadalene	t	0.2	0.4	0.2	0.8	0.8	0.7	
1958	isopimara-8(14),15-diene	0.7	0.5	t	t	0.1	0.5	0.4	
2009	manool oxide	0.2	0.1	t	0.1	0.3	0.3	1.5	0.1
2055	abietatriene	1.4	1.5	0.2	0.4	0.6	1.0	1.0	0.2
2087	abietadiene	0.5	1.2	0.3	0.5	0.5	0.8	0.3	1.2
2105	abienol, iso-, FW 290	5.3	8.8	1.8	1.6	3.1	4.2	9.6	(3.1)
2132	nezukol	-	-	-	-	-	-	-	0.2
2184	sandaracopimarinal	0.2	t	-	-	t	0.5	0.2	
2208	cis-totarol, methyl ether	0.7	1.0	t	0.3	0.3	0.8	0.5	0.1
2237	trans-totarol, methyl ether	1.2	0.7	0.2	0.4	0.7	1.3	1.2	
2269	sandaracopimarinol	t	t	t	-	0.2	0.6	0.2	
2282	sempervirol	1.0	0.7	t	0.3	0.8	2.0	1.0	2.4
2314	trans-totarol	9.6	5.3	1.0	1.9	5.8	16.8	7.8	11.7
2331	trans-ferruginol	0.3	0.3	t	t	0.2	0.3	0.1	0.6
2341	diterpene, 301,205,219, FW316	0.8	0.4	t	0.2	0.4	0.9	0.6	0.7
2364	diterpene, 190,175,277, FW316	1.0	0.5	t	0.2	0.6	1.8	0.8	0.9
2432	diterpene, 285,189,203, FW316	0.5	0.3	t	t	0.3	0.9	0.4	

KI = Kovat's Index (linear by temperature programming) om J & W DB-5 column. Values less than 0.05% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

Table 2. Correlation among 15 major terpenoids. APN = α-pinene, SBN = sabinene, MYR = myrcene, GTR = γ-terpinene, TRP = terpinolene, UMB = umbellulone, 4TR = terpinen-4-ol, GCD = γ-cadinene, DCD = γ-cadinene, ELO = elemol (+ hedycaryol), BUD = β-eudesmol, IAB = iso-abienol, TME = trans-totarol, methyl ether, TTTL = trans-totarol, GR4 = germacrene D-4-ol.

	APN	SBN	MYR	GTR	TRP	UMB	4TR	GCD	DCD	ELO	BUD	IAB	TME	TTL	GR4
APN	1.00	-.67	.62	-.47	.59	.35	-.62	.21	.18	-.57	-.56	.46	.44	.22	-.15
SBN	-.67	1.00	-.24	0.83	-.36	-.31	.91	-.12	-.13	.22	.08	.-.50	-.76	-.66	.19
MYR	.62	-.24	1.00	.06	.80	.57	-.15	.26	.28	-.81	-.83	.25	.22	-.05	-.04
GTR	-.47	.83	.06	1.00	-.11	-.08	.94	-.06	.02	-.04	-.13	-.43	-.62	-.62	.18
TRP	.59	-.36	.80	-.11	1.00	.42	-.29	.24	.25	-.65	-.65	.22	.34	.07	-.08
UMB	.35	-.31	.57	-.08	.42	1.00	-.12	.40	.47	-.60	-.54	.30	.13	-.08	.33
4TR	-.62	.91	-.15	.94	-.29	-.12	1.00	-.03	.02	.12	.04	-.50	-.72	-.69	.28
GCD	.21	-.12	.26	-.06	.24	.40	-.03	1.00	.92	-.41	-.39	.31	-.14	-.36	.74
DCD	.18	-.13	.28	.02	.25	.47	.02	.92	1.00	-.48	-.44	.26	-.08	-.27	.81
ELO	-.57	.22	-.81	-.04	-.65	-.60	.12	-.41	-.48	1.00	.92	-.35	-.24	.01	-.16
BUD	-.56	.08	-.83	-.13	-.65	-.54	.04	-.39	-.44	.92	1.00	-.30	-.08	.17	-.16
IAB	.46	-.50	.25	-.43	.22	.30	-.50	.31	.26	-.35	-.30	1.00	.38	.17	.05
TME	.44	-.76	.22	-.62	.34	.13	-.72	-.14	-.08	-.24	-.08	.38	1.00	.72	-.43
TTL	.22	-.66	-.05	-.62	.07	-.08	-.69	-.36	-.27	.01	.17	.17	.72	1.00	-.48
GR4	-.15	.19	-.04	.18	-.08	.33	.28	.74	.81	-.16	-.16	.05	-.43	-.48	1.00

Table 3. Individuals, sorted by population, with unusual plants grouped with most similar population by Minimum spanning network analysis (Fig. 3) and 15 terpenoids grouped by correlation.

	4TR	GTR	SBN	APN	MYR	TRP	UMB	GCD	DCD	ELO	BUD	IAB	TTM	TTT	GR4
BLJ Bailongjiang River Gansu, 3 atypical oils: B1, B3, B7															
B1 15754	3.0	1.1	15.8	2.2	1.3	0.9	0.05	1.3	3.3	21.7	6.2	1.1	0.4	5.6	4.0
B3 15756	1.0	0.3	1.0	3.7	0.6	0.5	0.05	0.4	1.8	14.1	9.0	4.2	1.3	16.8	1.1
B7 15760	2.0	0.9	9.3	5.5	1.4	0.8	0.03	0.6	0.6	14.8	5.4	9.6	1.2	7.8	0.02
BLJ Bailongjiang River, Gansu, typical oils															
B2 15755	5.4	2.4	31.5	4.1	2.0	1.3	0.05	0.2	1.3	11.3	3.3	0.8	0.6	7.2	1.3
B5 15758	4.8	2.4	30.8	6.6	2.3	1.5	0.03	0.4	1.2	10.9	2.6	3.1	0.7	5.9	1.0
B9 15762	6.0	2.8	31.8	3.2	2.2	1.4	0.02	0.3	0.4	12.7	3.2	3.8	0.6	3.8	0.02
B11 15764	6.4	2.3	21.7	4.2	2.0	1.5	2.6	0.4	1.1	14.2	5.9	1.6	0.5	6.0	0.8
B12 15765	4.8	2.0	21.6	9.2	2.0	1.4	1.8	0.4	0.9	16.0	5.0	3.1	0.6	5.4	0.8
B6 15759	3.5	1.7	18.8	7.8	1.9	1.2	0.02	0.4	1.6	14.8	4.5	1.6	0.7	6.5	1.0
B4 15757	3.7	1.7	14.7	11.7	2.2	1.6	0.9	0.9	2.1	10.8	3.1	2.8	0.8	5.3	1.6
B8 15761	4.3	1.9	13.1	4.4	2.3	1.6	0.4	0.2	0.9	15.1	7.5	3.5	1.2	6.4	0.6
B10 15763	4.6	2.2	22.3	3.4	1.9	1.7	1.2	0.3	1.0	9.7	2.9	8.1	0.8	8.9	1.0
DDH Daduhe River, Sichuan, 6 'hybrid' oils, more similar to BLJ oils															
D9 15740	6.2	2.5	35.7	4.5	2.2	1.3	0.05	0.6	0.9	3.3	1.4	1.6	0.4	1.9	0.02
D7 15738	6.6	2.9	28.8	6.4	2.3	1.6	3.2	1.3	3.3	4.8	2.0	1.8	0.2	1.0	4.3
D11 15742	7.0	3.2	33.2	10.3	2.5	1.5	0.05	1.2	3.3	4.1	1.7	1.4	0.3	2.4	2.4
D2 15733	6.4	3.6	23.0	4.7	3.1	2.3	2.6	1.4	4.9	0.05	0.05	3.6	0.9	6.2	3.0
D3 15734	5.7	2.7	22.7	8.4	3.8	2.4	5.3	1.1	3.6	0.05	0.05	3.8	0.6	4.7	2.7
D12 15743	5.9	2.3	25.2	5.6	2.4	1.6	3.7	1.6	4.5	0.05	0.10	3.5	0.6	3.9	5.8
DDH Daduhe River, Sichuan, 6 typical oils															
D10 15741	2.8	1.4	9.8	18.5	2.6	1.8	5.0	1.5	3.9	6.2	1.8	6.3	0.20	3.7	3.4
D4 15735	2.2	1.1	7.8	17.6	2.5	1.6	0.7	1.5	3.9	6.0	2.4	10.5	1.1	5.3	2.5
D6 15737	3.9	1.5	11.5	11.6	2.2	1.7	4.3	1.8	5.3	4.9	1.4	7.9	0.8	4.2	4.0
D8 15739	4.1	1.8	14.4	8.3	2.9	2.1	8.8	1.8	4.1	3.9	2.1	5.4	1.0	3.0	3.2
D5 15736	3.4	1.6	9.0	17.2	3.1	2.0	2.7	2.4	5.5	0.05	0.2	6.0	1.2	6.1	2.7
D1 15732	4.1	2.2	15.6	14.1	2.9	2.0	3.5	1.0	3.7	0.1	0.1	11.3	0.9	6.5	2.5
MJR Minjiang River, Sichuan, lower elevation, near Gansu, typical oils															
M9 15752	1.2	0.6	0.6	25.1	2.6	2.0	2.3	0.05	0.9	7.0	2.4	2.5	1.6	10.4	0.03
M8 15751	5.1	3.1	13.3	19.4	3.1	2.7	1.4	0.4	1.3	5.2	1.5	2.9	0.8	5.1	0.10
M10 15753	3.8	2.2	14.3	9.7	3.0	2.4	4.0	0.03	1.1	3.7	1.5	3.8	1.0	8.0	0.03
M6 15749	3.2	1.8	8.8	18.3	3.1	0.6	4.4	0.4	1.4	0.4	0.4	5.5	0.9	10.4	0.4
M1 15744	1.7	1.3	8.2	18.9	3.9	2.6	3.5	0.3	1.0	0.4	0.05	6.5	1.1	8.9	0.3
M4 15747	1.0	0.6	2.7	15.7	3.3	2.9	3.2	0.2	1.3	0.5	0.05	5.3	1.4	10.1	0.2
M7 15750	1.7	1.0	2.7	17.4	2.7	2.8	3.9	0.7	3.0	0.2	0.03	4.2	1.6	10.6	0.30
M2 15745	0.8	0.5	2.8	29.6	3.2	3.0	3.3	1.4	2.7	0.05	0.02	11.8	1.0	6.2	1.7
M3 15746	1.0	0.7	2.4	18.2	3.7	2.6	4.2	1.7	3.9	0.05	0.02	9.0	1.2	9.4	1.2
M5 15748	1.4	1.1	5.2	19.6	3.7	3.8	1.8	1.7	3.5	0.03	0.02	3.7	1.0	9.7	1.2

Table 4. Individuals, sorted by sabinene concentration with unusual plants grouped with most similar population and 15 terpenoids grouped by correlation. For compound abbreviations, see Table 3.

	4TR	GTR	SBN	APN	MYR	TRP	UMB	GCD	DCD	ELO	BUD	IAB	TTM	TTT	GR4
D9 15740	6.2	2.5	35.7	4.5	2.2	1.3	0.05	0.6	0.9	3.3	1.4	1.6	0.4	1.9	0.02
D11 15742	7.0	3.2	33.2	10.3	2.5	1.5	0.05	1.2	3.3	4.1	1.7	1.4	0.3	2.4	2.4
B9 15762	6.0	2.8	31.8	3.2	2.2	1.4	0.02	0.3	0.4	12.7	3.2	3.8	0.6	3.8	0.02
B2 15755	5.4	2.4	31.5	4.1	2.0	1.3	0.05	0.2	1.3	11.3	3.3	0.8	0.6	7.2	1.3
B5 15758	4.8	2.4	30.8	6.6	2.3	1.5	0.03	0.4	1.2	10.9	2.6	3.1	0.7	5.9	1.0
D7 15738	6.6	2.9	28.8	6.4	2.3	1.6	3.2	1.3	3.3	4.8	2.0	1.8	0.2	1.0	4.3
D12 15743	5.9	2.3	25.2	5.6	2.4	1.6	3.7	1.6	4.5	0.05	0.10	3.5	0.6	3.9	5.8
D2 15733	6.4	3.6	23.0	4.7	3.1	2.3	2.6	1.4	4.9	0.05	0.05	3.6	0.9	6.2	3.0
D3 15734	5.7	2.7	22.7	8.4	3.8	2.4	5.3	1.1	3.6	0.05	0.05	3.8	0.6	4.7	2.7
B10 15763	4.6	2.2	22.3	3.4	1.9	1.7	1.2	0.3	1.0	9.7	2.9	8.1	0.8	8.9	1.0
B11 15764	6.4	2.3	21.7	4.2	2.0	1.5	2.6	0.4	1.1	14.2	5.9	1.6	0.5	6.0	0.8
B12 15765	4.8	2.0	21.6	9.2	2.0	1.4	1.8	0.4	0.9	16.0	5.0	3.1	0.6	5.4	0.8
B6 15759	3.5	1.7	18.8	7.8	1.9	1.2	0.02	0.4	1.6	14.8	4.5	1.6	0.7	6.5	1.0
B1 15754	3.0	1.1	15.8	2.2	1.3	0.9	0.05	1.3	3.3	21.7	6.2	1.1	0.4	5.6	4.0
D1 15732	4.1	2.2	15.6	14.1	2.9	2.0	3.5	1.0	3.7	0.1	0.1	11.3	0.9	6.5	2.5
B4 15757	3.7	1.7	14.7	11.7	2.2	1.6	0.9	0.9	2.1	10.8	3.1	2.8	0.8	5.3	1.6
D8 15739	4.1	1.8	14.4	8.3	2.9	2.1	8.8	1.8	4.1	3.9	2.1	5.4	1.0	3.0	3.2
M10 15753	3.8	2.2	14.3	9.7	3.0	2.4	4.0	0.03	1.1	3.7	1.5	3.8	1.0	8.0	0.03
M8 15751	5.1	3.1	13.3	19.4	3.1	2.7	1.4	0.4	1.3	5.2	1.5	2.9	0.8	5.1	0.10
B8 15761	4.3	1.9	13.1	4.4	2.3	1.6	0.4	0.2	0.9	15.1	7.5	3.5	1.2	6.4	0.6
D6 15737	3.9	1.5	11.5	11.6	2.2	1.7	4.3	1.8	5.3	4.9	1.4	7.9	0.8	4.2	4.0
D10 15741	2.8	1.4	9.8	18.5	2.6	1.8	5.0	1.5	3.9	6.2	1.8	6.3	0.20	3.7	3.4
B7 15760	2.0	0.9	9.3	5.5	1.4	0.8	0.03	0.6	0.6	14.8	5.4	9.6	1.2	7.8	0.02
D5 15736	3.4	1.6	9.0	17.2	3.1	2.0	2.7	2.4	5.5	0.05	0.2	6.0	1.2	6.1	2.7
M6 15749	3.2	1.8	8.8	18.3	3.1	0.6	4.4	0.4	1.4	0.4	0.4	5.5	0.9	10.4	0.4
M1 15744	1.7	1.3	8.2	18.9	3.9	2.6	3.5	0.3	1.0	0.4	0.05	6.5	1.1	8.9	0.3
D4 15735	2.2	1.1	7.8	17.6	2.5	1.6	0.7	1.5	3.9	6.0	2.4	10.5	1.1	5.3	2.5
M5 15748	1.4	1.1	5.2	19.6	3.7	3.8	1.8	1.7	3.5	0.03	0.02	3.7	1.0	9.7	1.2
M2 15745	0.8	0.5	2.8	29.6	3.2	3.0	3.3	1.4	2.7	0.05	0.02	11.8	1.0	6.2	1.7
M4 15747	1.0	0.6	2.7	15.7	3.3	2.9	3.2	0.2	1.3	0.5	0.05	5.3	1.4	10.1	0.2
M7 15750	1.7	1.0	2.7	17.4	2.7	2.8	3.9	0.7	3.0	0.2	0.03	4.2	1.6	10.6	0.30
M3 15746	1.0	0.7	2.4	18.2	3.7	2.6	4.2	1.7	3.9	0.05	0.02	9.0	1.2	9.4	1.2
B3 15756	1.0	0.3	1.0	3.7	0.6	0.5	0.05	0.4	1.8	14.1	9.0	4.2	1.3	16.8	1.1
M9 15752	1.2	0.6	0.6	25.1	2.6	2.0	2.3	0.05	0.9	7.0	2.4	2.5	1.6	10.4	0.03

Table 5. Individuals, sorted by elemol/ hedycaryol concentration with unusual plants grouped with most similar population and 15 terpenoids grouped by correlation. For compound abbreviations, see Table 3.

	4TR	GTR	SBN	APN	MYR	TRP	UMB	GCD	DCD	ELO	BUD	IAB	TTM	TTT	GR4
B1 15754	3.0	1.1	15.8	2.2	1.3	0.9	0.05	1.3	3.3	21.7	6.2	1.1	0.4	5.6	4.0
B12 15765	4.8	2.0	21.6	9.2	2.0	1.4	1.8	0.4	0.9	16.0	5.0	3.1	0.6	5.4	0.8
B8 15761	4.3	1.9	13.1	4.4	2.3	1.6	0.4	0.2	0.9	15.1	7.5	3.5	1.2	6.4	0.6
B6 15759	3.5	1.7	18.8	7.8	1.9	1.2	0.02	0.4	1.6	14.8	4.5	1.6	0.7	6.5	1.0
B7 15760	2.0	0.9	9.3	5.5	1.4	0.8	0.03	0.6	0.6	14.8	5.4	9.6	1.2	7.8	0.02
B11 15764	6.4	2.3	21.7	4.2	2.0	1.5	2.6	0.4	1.1	14.2	5.9	1.6	0.5	6.0	0.8
B3 15756	1.0	0.3	1.0	3.7	0.6	0.5	0.05	0.4	1.8	14.1	9.0	4.2	1.3	16.8	1.1
B9 15762	6.0	2.8	31.8	3.2	2.2	1.4	0.02	0.3	0.4	12.7	3.2	3.8	0.6	3.8	0.02
B2 15755	5.4	2.4	31.5	4.1	2.0	1.3	0.05	0.2	1.3	11.3	3.3	0.8	0.6	7.2	1.3
B5 15758	4.8	2.4	30.8	6.6	2.3	1.5	0.03	0.4	1.2	10.9	2.6	3.1	0.7	5.9	1.0
B4 15757	3.7	1.7	14.7	11.7	2.2	1.6	0.9	0.9	2.1	10.8	3.1	2.8	0.8	5.3	1.6
B10 15763	4.6	2.2	22.3	3.4	1.9	1.7	1.2	0.3	1.0	9.7	2.9	8.1	0.8	8.9	1.0
M9 15752	1.2	0.6	0.6	25.1	2.6	2.0	2.3	0.05	0.9	7.0	2.4	2.5	1.6	10.4	0.03
D10 15741	2.8	1.4	9.8	18.5	2.6	1.8	5.0	1.5	3.9	6.2	1.8	6.3	0.20	3.7	3.4
D4 15735	2.2	1.1	7.8	17.6	2.5	1.6	0.7	1.5	3.9	6.0	2.4	10.5	1.1	5.3	2.5
M8 15751	5.1	3.1	13.3	19.4	3.1	2.7	1.4	0.4	1.3	5.2	1.5	2.9	0.8	5.1	0.10
D6 15737	3.9	1.5	11.5	11.6	2.2	1.7	4.3	1.8	5.3	4.9	1.4	7.9	0.8	4.2	4.0
D7 15738	6.6	2.9	28.8	6.4	2.3	1.6	3.2	1.3	3.3	4.8	2.0	1.8	0.2	1.0	4.3
D11 15742	7.0	3.2	33.2	10.3	2.5	1.5	0.05	1.2	3.3	4.1	1.7	1.4	0.3	2.4	2.4
D8 15739	4.1	1.8	14.4	8.3	2.9	2.1	8.8	1.8	4.1	3.9	2.1	5.4	1.0	3.0	3.2
M10 15753	3.8	2.2	14.3	9.7	3.0	2.4	4.0	0.03	1.1	3.7	1.5	3.8	1.0	8.0	0.03
D9 15740	6.2	2.5	35.7	4.5	2.2	1.3	0.05	0.6	0.9	3.3	1.4	1.6	0.4	1.9	0.02
M4 15747	1.0	0.6	2.7	15.7	3.3	2.9	3.2	0.2	1.3	0.5	0.05	5.3	1.4	10.1	0.2
M6 15749	3.2	1.8	8.8	18.3	3.1	0.6	4.4	0.4	1.4	0.4	0.4	5.5	0.9	10.4	0.4
M1 15744	1.7	1.3	8.2	18.9	3.9	2.6	3.5	0.3	1.0	0.4	0.05	6.5	1.1	8.9	0.3
M7 15750	1.7	1.0	2.7	17.4	2.7	2.8	3.9	0.7	3.0	0.2	0.03	4.2	1.6	10.6	0.30
D1 15732	4.1	2.2	15.6	14.1	2.9	2.0	3.5	1.0	3.7	0.1	0.1	11.3	0.9	6.5	2.5
D12 15743	5.9	2.3	25.2	5.6	2.4	1.6	3.7	1.6	4.5	0.05	0.10	3.5	0.6	3.9	5.8
D2 15733	6.4	3.6	23.0	4.7	3.1	2.3	2.6	1.4	4.9	0.05	0.05	3.6	0.9	6.2	3.0
D3 15734	5.7	2.7	22.7	8.4	3.8	2.4	5.3	1.1	3.6	0.05	0.05	3.8	0.6	4.7	2.7
D5 15736	3.4	1.6	9.0	17.2	3.1	2.0	2.7	2.4	5.5	0.05	0.2	6.0	1.2	6.1	2.7
M2 15745	0.8	0.5	2.8	29.6	3.2	3.0	3.3	1.4	2.7	0.05	0.02	11.8	1.0	6.2	1.7
M3 15746	1.0	0.7	2.4	18.2	3.7	2.6	4.2	1.7	3.9	0.05	0.02	9.0	1.2	9.4	1.2
M5 15748	1.4	1.1	5.2	19.6	3.7	3.8	1.8	1.7	3.5	0.03	0.02	3.7	1.0	9.7	1.2

Table 6. Individuals, sorted by umbellulone (UMB) concentration with unusual plants grouped with most similar population and 15 terpenoids grouped by correlation. For compound abbreviations, see Table 3.

	4TR	GTR	SBN	APN	MYR	TRP	UMB	GCD	DCD	ELO	BUD	IAB	TTM	TTT	GR4
D8 15739	4.1	1.8	14.4	8.3	2.9	2.1	8.8	1.8	4.1	3.9	2.1	5.4	1.0	3.0	3.2
D3 15734	5.7	2.7	22.7	8.4	3.8	2.4	5.3	1.1	3.6	0.05	0.05	3.8	0.6	4.7	2.7
D10 15741	2.8	1.4	9.8	18.5	2.6	1.8	5.0	1.5	3.9	6.2	1.8	6.3	0.20	3.7	3.4
M6 15749	3.2	1.8	8.8	18.3	3.1	0.6	4.4	0.4	1.4	0.4	0.4	5.5	0.9	10.4	0.4
D6 15737	3.9	1.5	11.5	11.6	2.2	1.7	4.3	1.8	5.3	4.9	1.4	7.9	0.8	4.2	4.0
M3 15746	1.0	0.7	2.4	18.2	3.7	2.6	4.2	1.7	3.9	0.05	0.02	9.0	1.2	9.4	1.2
M10 15753	3.8	2.2	14.3	9.7	3.0	2.4	4.0	0.03	1.1	3.7	1.5	3.8	1.0	8.0	0.03
M7 15750	1.7	1.0	2.7	17.4	2.7	2.8	3.9	0.7	3.0	0.2	0.03	4.2	1.6	10.6	0.30
D12 15743	5.9	2.3	25.2	5.6	2.4	1.6	3.7	1.6	4.5	0.05	0.10	3.5	0.6	3.9	5.8
D1 15732	4.1	2.2	15.6	14.1	2.9	2.0	3.5	1.0	3.7	0.1	0.1	11.3	0.9	6.5	2.5
M1 15744	1.7	1.3	8.2	18.9	3.9	2.6	3.5	0.3	1.0	0.4	0.05	6.5	1.1	8.9	0.3
M2 15745	0.8	0.5	2.8	29.6	3.2	3.0	3.3	1.4	2.7	0.05	0.02	11.8	1.0	6.2	1.7
D7 15738	6.6	2.9	28.8	6.4	2.3	1.6	3.2	1.3	3.3	4.8	2.0	1.8	0.2	1.0	4.3
M4 15747	1.0	0.6	2.7	15.7	3.3	2.9	3.2	0.2	1.3	0.5	0.05	5.3	1.4	10.1	0.2
D5 15736	3.4	1.6	9.0	17.2	3.1	2.0	2.7	2.4	5.5	0.05	0.2	6.0	1.2	6.1	2.7
B11 15764	6.4	2.3	21.7	4.2	2.0	1.5	2.6	0.4	1.1	14.2	5.9	1.6	0.5	6.0	0.8
D2 15733	6.4	3.6	23.0	4.7	3.1	2.3	2.6	1.4	4.9	0.05	0.05	3.6	0.9	6.2	3.0
M9 15752	1.2	0.6	0.6	25.1	2.6	2.0	2.3	0.05	0.9	7.0	2.4	2.5	1.6	10.4	0.03
B12 15765	4.8	2.0	21.6	9.2	2.0	1.4	1.8	0.4	0.9	16.0	5.0	3.1	0.6	5.4	0.8
M5 15748	1.4	1.1	5.2	19.6	3.7	3.8	1.8	1.7	3.5	0.03	0.02	3.7	1.0	9.7	1.2
M8 15751	5.1	3.1	13.3	19.4	3.1	2.7	1.4	0.4	1.3	5.2	1.5	2.9	0.8	5.1	0.10
B10 15763	4.6	2.2	22.3	3.4	1.9	1.7	1.2	0.3	1.0	9.7	2.9	8.1	0.8	8.9	1.0
B4 15757	3.7	1.7	14.7	11.7	2.2	1.6	0.9	0.9	2.1	10.8	3.1	2.8	0.8	5.3	1.6
D4 15735	2.2	1.1	7.8	17.6	2.5	1.6	0.7	1.5	3.9	6.0	2.4	10.5	1.1	5.3	2.5
B8 15761	4.3	1.9	13.1	4.4	2.3	1.6	0.4	0.2	0.9	15.1	7.5	3.5	1.2	6.4	0.6
B1 15754	3.0	1.1	15.8	2.2	1.3	0.9	0.05	1.3	3.3	21.7	6.2	1.1	0.4	5.6	4.0
B3 15756	1.0	0.3	1.0	3.7	0.6	0.5	0.05	0.4	1.8	14.1	9.0	4.2	1.3	16.8	1.1
D9 15740	6.2	2.5	35.7	4.5	2.2	1.3	0.05	0.6	0.9	3.3	1.4	1.6	0.4	1.9	0.02
B2 15755	5.4	2.4	31.5	4.1	2.0	1.3	0.05	0.2	1.3	11.3	3.3	0.8	0.6	7.2	1.3
D11 15742	7.0	3.2	33.2	10.3	2.5	1.5	0.05	1.2	3.3	4.1	1.7	1.4	0.3	2.4	2.4
B7 15760	2.0	0.9	9.3	5.5	1.4	0.8	0.03	0.6	0.6	14.8	5.4	9.6	1.2	7.8	0.02
B5 15758	4.8	2.4	30.8	6.6	2.3	1.5	0.03	0.4	1.2	10.9	2.6	3.1	0.7	5.9	1.0
B9 15762	6.0	2.8	31.8	3.2	2.2	1.4	0.02	0.3	0.4	12.7	3.2	3.8	0.6	3.8	0.02
B6 15759	3.5	1.7	18.8	7.8	1.9	1.2	0.02	0.4	1.6	14.8	4.5	1.6	0.7	6.5	1.0

Table 7. Individuals, sorted by germacrene D-4-ol (GR4) concentration with unusual plants grouped with most similar population and 15 terpenoids grouped by correlation. For compound abbreviations, see Table 3.

	4TR	GTR	SBN	APN	MYR	TRP	UMB	GCD	DCD	ELO	BUD	IAB	TTM	TTT	GR4
D12 15743	5.9	2.3	25.2	5.6	2.4	1.6	3.7	1.6	4.5	0.05	0.10	3.5	0.6	3.9	5.8
D7 15738	6.6	2.9	28.8	6.4	2.3	1.6	3.2	1.3	3.3	4.8	2.0	1.8	0.2	1.0	4.3
B1 15754	3.0	1.1	15.8	2.2	1.3	0.9	0.05	1.3	3.3	21.7	6.2	1.1	0.4	5.6	4.0
D6 15737	3.9	1.5	11.5	11.6	2.2	1.7	4.3	1.8	5.3	4.9	1.4	7.9	0.8	4.2	4.0
D10 15741	2.8	1.4	9.8	18.5	2.6	1.8	5.0	1.5	3.9	6.2	1.8	6.3	0.20	3.7	3.4
D8 15739	4.1	1.8	14.4	8.3	2.9	2.1	8.8	1.8	4.1	3.9	2.1	5.4	1.0	3.0	3.2
D2 15733	6.4	3.6	23.0	4.7	3.1	2.3	2.6	1.4	4.9	0.05	0.05	3.6	0.9	6.2	3.0
D3 15734	5.7	2.7	22.7	8.4	3.8	2.4	5.3	1.1	3.6	0.05	0.05	3.8	0.6	4.7	2.7
D5 15736	3.4	1.6	9.0	17.2	3.1	2.0	2.7	2.4	5.5	0.05	0.2	6.0	1.2	6.1	2.7
D4 15735	2.2	1.1	7.8	17.6	2.5	1.6	0.7	1.5	3.9	6.0	2.4	10.5	1.1	5.3	2.5
D1 15732	4.1	2.2	15.6	14.1	2.9	2.0	3.5	1.0	3.7	0.1	0.1	11.3	0.9	6.5	2.5
D11 15742	7.0	3.2	33.2	10.3	2.5	1.5	0.05	1.2	3.3	4.1	1.7	1.4	0.3	2.4	2.4
M2 15745	0.8	0.5	2.8	29.6	3.2	3.0	3.3	1.4	2.7	0.05	0.02	11.8	1.0	6.2	1.7
B4 15757	3.7	1.7	14.7	11.7	2.2	1.6	0.9	0.9	2.1	10.8	3.1	2.8	0.8	5.3	1.6
B2 15755	5.4	2.4	31.5	4.1	2.0	1.3	0.05	0.2	1.3	11.3	3.3	0.8	0.6	7.2	1.3
M3 15746	1.0	0.7	2.4	18.2	3.7	2.6	4.2	1.7	3.9	0.05	0.02	9.0	1.2	9.4	1.2
M5 15748	1.4	1.1	5.2	19.6	3.7	3.8	1.8	1.7	3.5	0.03	0.02	3.7	1.0	9.7	1.2
B3 15756	1.0	0.3	1.0	3.7	0.6	0.5	0.05	0.4	1.8	14.1	9.0	4.2	1.3	16.8	1.1
B6 15759	3.5	1.7	18.8	7.8	1.9	1.2	0.02	0.4	1.6	14.8	4.5	1.6	0.7	6.5	1.0
B5 15758	4.8	2.4	30.8	6.6	2.3	1.5	0.03	0.4	1.2	10.9	2.6	3.1	0.7	5.9	1.0
B10 15763	4.6	2.2	22.3	3.4	1.9	1.7	1.2	0.3	1.0	9.7	2.9	8.1	0.8	8.9	1.0
B12 15765	4.8	2.0	21.6	9.2	2.0	1.4	1.8	0.4	0.9	16.0	5.0	3.1	0.6	5.4	0.8
B11 15764	6.4	2.3	21.7	4.2	2.0	1.5	2.6	0.4	1.1	14.2	5.9	1.6	0.5	6.0	0.8
B8 15761	4.3	1.9	13.1	4.4	2.3	1.6	0.4	0.2	0.9	15.1	7.5	3.5	1.2	6.4	0.6
M6 15749	3.2	1.8	8.8	18.3	3.1	0.6	4.4	0.4	1.4	0.4	0.4	5.5	0.9	10.4	0.4
M1 15744	1.7	1.3	8.2	18.9	3.9	2.6	3.5	0.3	1.0	0.4	0.05	6.5	1.1	8.9	0.3
M7 15750	1.7	1.0	2.7	17.4	2.7	2.8	3.9	0.7	3.0	0.2	0.03	4.2	1.6	10.6	0.30
M4 15747	1.0	0.6	2.7	15.7	3.3	2.9	3.2	0.2	1.3	0.5	0.05	5.3	1.4	10.1	0.2
M8 15751	5.1	3.1	13.3	19.4	3.1	2.7	1.4	0.4	1.3	5.2	1.5	2.9	0.8	5.1	0.10
M9 15752	1.2	0.6	0.6	25.1	2.6	2.0	2.3	0.05	0.9	7.0	2.4	2.5	1.6	10.4	0.03
M10 15753	3.8	2.2	14.3	9.7	3.0	2.4	4.0	0.03	1.1	3.7	1.5	3.8	1.0	8.0	0.03
B7 15760	2.0	0.9	9.3	5.5	1.4	0.8	0.03	0.6	0.6	14.8	5.4	9.6	1.2	7.8	0.02
B9 15762	6.0	2.8	31.8	3.2	2.2	1.4	0.02	0.3	0.4	12.7	3.2	3.8	0.6	3.8	0.02
D9 15740	6.2	2.5	35.7	4.5	2.2	1.3	0.05	0.6	0.9	3.3	1.4	1.6	0.4	1.9	0.02

Long distance gene flow facilitated by bird-dispersed seeds in wind-pollinated species: A story of hybridization and introgression between *Juniperus ashei* and *J. ovata* told by nrDNA and cpDNA

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ABSTRACT

nrDNA and cpDNA were sequenced of *J. ashei* and *J. ovata* from populations throughout their ranges. No *J. ashei* populations were found to be pure in their nrDNA for every tree, however all *J. ashei* trees in every population contained only the pure *J. ashei* chloroplast type. Populations of *J. ovata* in trans-Pecos Texas were almost pure in both nrDNA and cp DNA. Several plants in the *J. ashei* range contained *J. ovata*-type nrDNA (White Cliffs, AR, 3/10); Ranger, TX (1/5); Waco, TX (1/12). Every *J ashei* population contained at least 1 plant with hybrid (heterozygous) nrDNA and 3 *J. ovata* populations contained putative hybrids (by nrDNA), but one population had only pure *J. ovata* trees. The presence of *ovata* germplasm within *J. ashei* populations seems best explained by long distance bird dispersal of *J. ovata* seeds (thence seedlings and *J. ovata* trees and hybrids) in the disjunct *J. ashei* populations. The reason for the absence of *ovata* paternal cp, which is distributed by pollen in *J. ashei* populations is not known. Judged by distribution of cp data, there is very little movement of cp genomes. In contrast, nrDNA polymorphisms indicate there is considerable gene flow between *J. ashei* and *J. ovata,* but primarily in the direction of *J. ovata* to *J. ashei* which may be explained by a combination of bird migration pattern and recurring and preferential F1-hybrid formation. *Published on-line www.phytologia.org Phytologia 102(2): 55-74 (June 24, 2020). ISSN 030319430.*

KEY WORDS: *Juniperus, J. ashei, J. ovata,* essential oils, distribution, Cupressaceae

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About 50 years ago, one of the authors (RPA) began a series of studies using leaf volatile oils examining variation in *J. ashei* populations (Adams, 1969). Surprisingly, it was discovered that the volatile leaf terpenoids had almost no variation (Adams and Turner 1970; Adams 1969; 1975; 1977) across hundreds of miles (Fig. 1) from the Texas hill country to the Ozark mountains in Arkansas-Missouri (the type locality is Sylamore, AR, Buchholz 1930). In fact, the gas chromatogram traces were so similar that one could lay the chromatograms on top of each other and see no differences. Over the ensuing 50 years of terpenoid analyses on all the 76 *Juniperus* species (Adams 2014), this uniformity has not been encountered in any other *Juniperus* species. However, several divergent populations were subsequently identified in the semi-arid trans-Pecos, Texas region (Ozona, Comstock) and adjacent Mexico (116, Sierra del Carman, Fig. 1). In addition, trees from New Braunfels, TX were found to have leaf terpenoid composition most similar to that found in the trans-Pecos/ Mexico region (Fig. 1). Follow-up research using RAPDs (Random Amplified Polymorphic DNAs) combined with morphological, and terpenoid differences led to the naming of the divergent populations as *J. ashei* var. *ovata* R. P. Adams (Adams and Baker 2007). Later use of DNA sequencing led to the recognition of *J. ashei* var. *ovata* at the specific level, *J. ovata* (R. P. Adams) R. P. Adams (Adams and Schwarzbach 2013). Thus, we will use *J. ovata* in place of the term 'divergent populations' of Adams (1977) throughout the remainder of this paper.

Comparing the leaf essential oils of *J. ashei* with *J. ovata*, revealed that they differ mostly in a quantitative fashion (Appdx. 1). —Camphor content is considerably larger in *J. ashei* (69.1%) than in *J. ovata* (53.5%, Appdx. 1). In contrast, bornyl acetate concentration is much larger in *J. ovata* (15.6%) than in *J. ashei* (6.3%)(Appdx. 1). In addition, four (non-trace) compounds differ qualitatively: trans-sabinene hydrate, trans-p-menth-2-en-1-ol, verbenone, and sandaracopimara-8(14),15-diene (Appdx.1) with all four occurring in *J. ashei*, but not *J. ovata*. Several other compounds differ quantitatively: α-pinene, myrcene, p-cymene, limonene, γ-terpinene, linalool, trans-carveol, carvone and elemol (Appdx. 1).

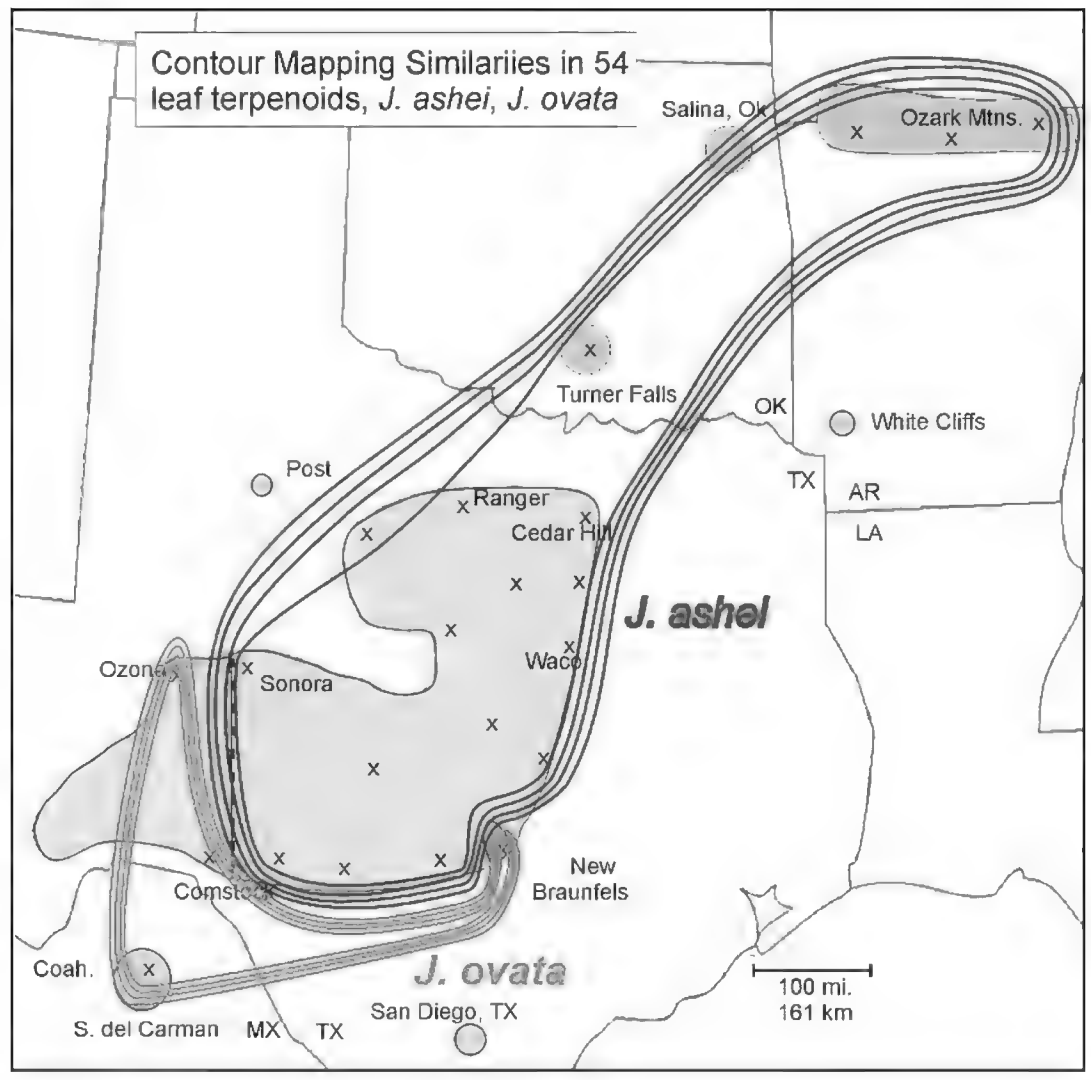

Figure 1. Contoured leaf oil similarities of populations of *J. ashei* and *J. ovata*. Adapted from Adams, 1977.

Juniperus ovata is generally easy to identify by the oval (elliptical) glands, especially on the whip (decurrent) leaves (Fig. 2). Notice hemispherical glands on *J. ashei* (Fig. 2, left) and the raised, oval to elongated glands on *J. ovata* (Fig. 2, right). It should be noted that a few nearly hemispherical glands are present on whip leaves of *J. ovata*. This is informative, as these characters can be used to distinguish *J. ovata* from *J. ashei*, yet exclude other nearby junipers species such as *J. monosperma* (Englem.) Sarg. *J. pinchotii* Sudw. and *J. coahuilensis*. (Mart.) Gaussen ex R. P. Adams. *Juniperus ovata* also has smaller cones, and more seeds per cone than *J. ashei*. (Table 1).

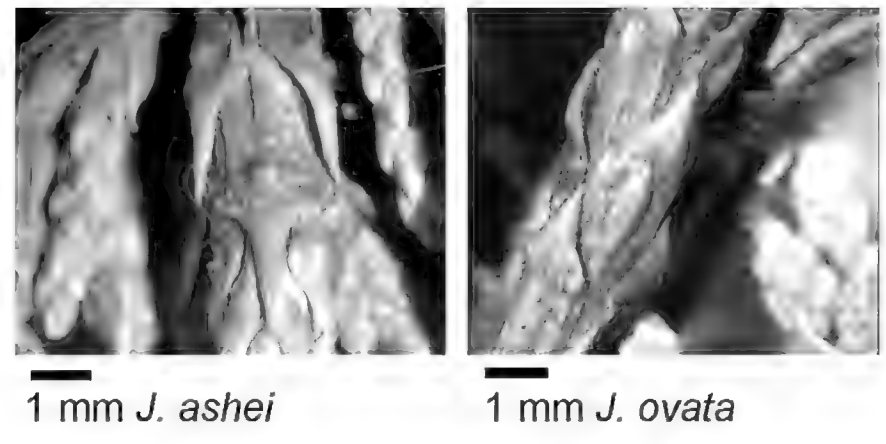

Figure 2. Comparison of whip leaf glands of *J. ashei* and *J. ovata*.

Table 1. Morphological differences between *J. ashei* and *J. ovata*.

Character	*J. ashei*	*J. ovata*
female cone diameter	larger (8)9(10) mm	smaller (5)6(8) mm
seeds per cone	fewer (1.01)	more (1.7)
seed size (L x W)	larger (16-27 mm^2)	smaller (13-16 mm^2)
whip leaf gland L/ sheath L	smaller ratio (0.20-0.30)	larger ratio (> 0.40)
whip leaf gland shape	hemispherical (1.0 - 1.5)	raised, oval to ellipse (2.0 - 2.5)
branching angle	narrow (45 - 40º)	wider (45 - 55º)

In the original study (Adams, 1977), the New Braunfels population of *Juniperus ovata* was represented by samples from 15 individuals from a single population 8 km west of New Braunfels. The nearest populations sampled (Adams, 1977) were at Bandera and Hyde (80 - 100 km w and nw of New Braunfels) and these had typical *J. ashei* leaf terpenoids. To determine if *J. ovata* extended further west, Adams (2008) obtained new samples from New Braunfels to the junction of US 281 and TX Hwy 46. Because tricyclene is fairly constant in *J. ashei*, by merely examining if the height of the α-pinene peak (that runs just after tricyclene on DB-5) is greater than tricyclene, one can determine that the oil is from *J. ovata*, whereas if α-pinene is less than tricyclene, the oil is from *J. ashei*.

Figure 3 shows that the samples taken along TX 46 from US 281 to near loop 337 are all low in α-pinene. This is typical for *J. ashei*. The samples from loop 337 (L) are high in α-pinene that is typical of *J. ovata*. The samples of *J. ovata* from the National Big Tree site (N) and nearby are uniformly high in α-pinene. Two of the samples along FM 482 are typical var. *ovata*, but the third sample is more like *J. ashei*.

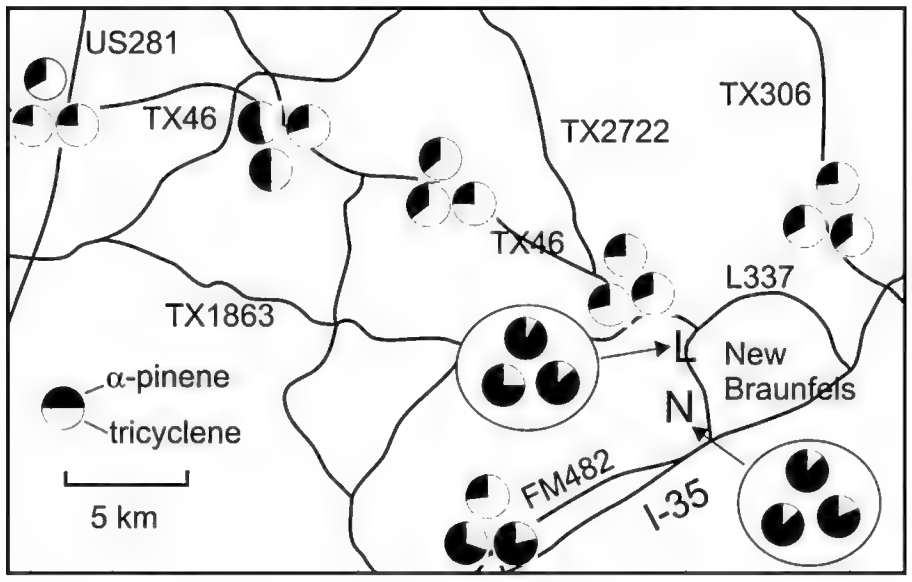

Figure 3. Distribution of *J. ashei* and *J. ovata* in the New Braunfels area based on the concentration of tricyclene and α-pinene. From Adams (2008).

Geographic variation in camphor and bornyl acetate show the same pattern (Fig. 4). However, at least one individual in both the FM 482 and the TX 306 populations appear to be intermediate between *J. ashei* and *J. ovata*, suggesting some hybridization between the taxa.

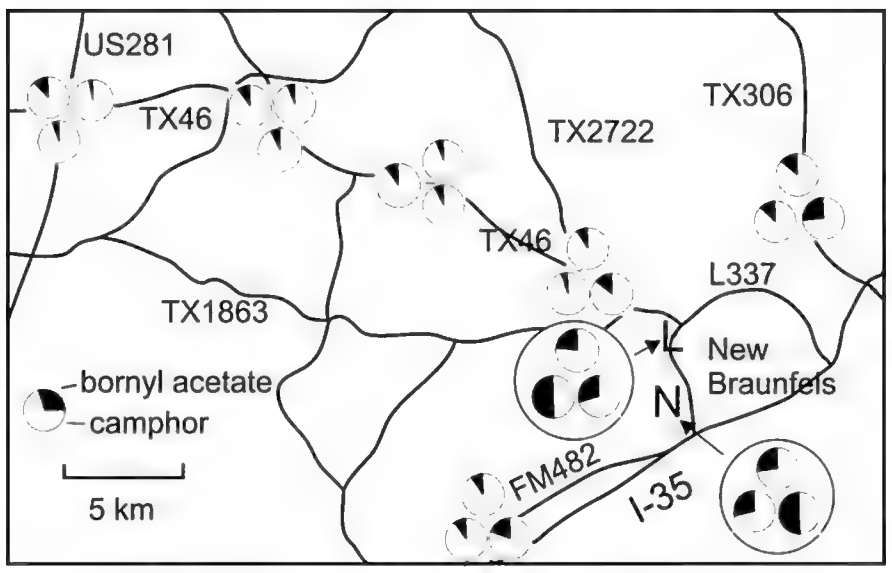

Figure 4. Distribution of *J. ashei* and *J. ovata* based on the concentration of bornyl acetate and camphor. From Adams (2008).

A preliminary study of nrDNA and cpDNA from the samples of Adams (2008) near New Braunfels, revealed a more complex pattern of hybridization and potential introgression than originally seen in the terpene analyses. The purpose of this paper is to present the results of a more exhaustive study of variation in nrDNA (ITS) and cpDNA throughout the ranges of *J. ashei* and *J. ovata*.

MATERIALS AND METHODS

Figure 5 shows the distribution of *J. ashei* and *J. ovata* with the populations sampled for this study. Additional samples where collected in the Comal Co. - New Braunfels areas (Fig. 6).

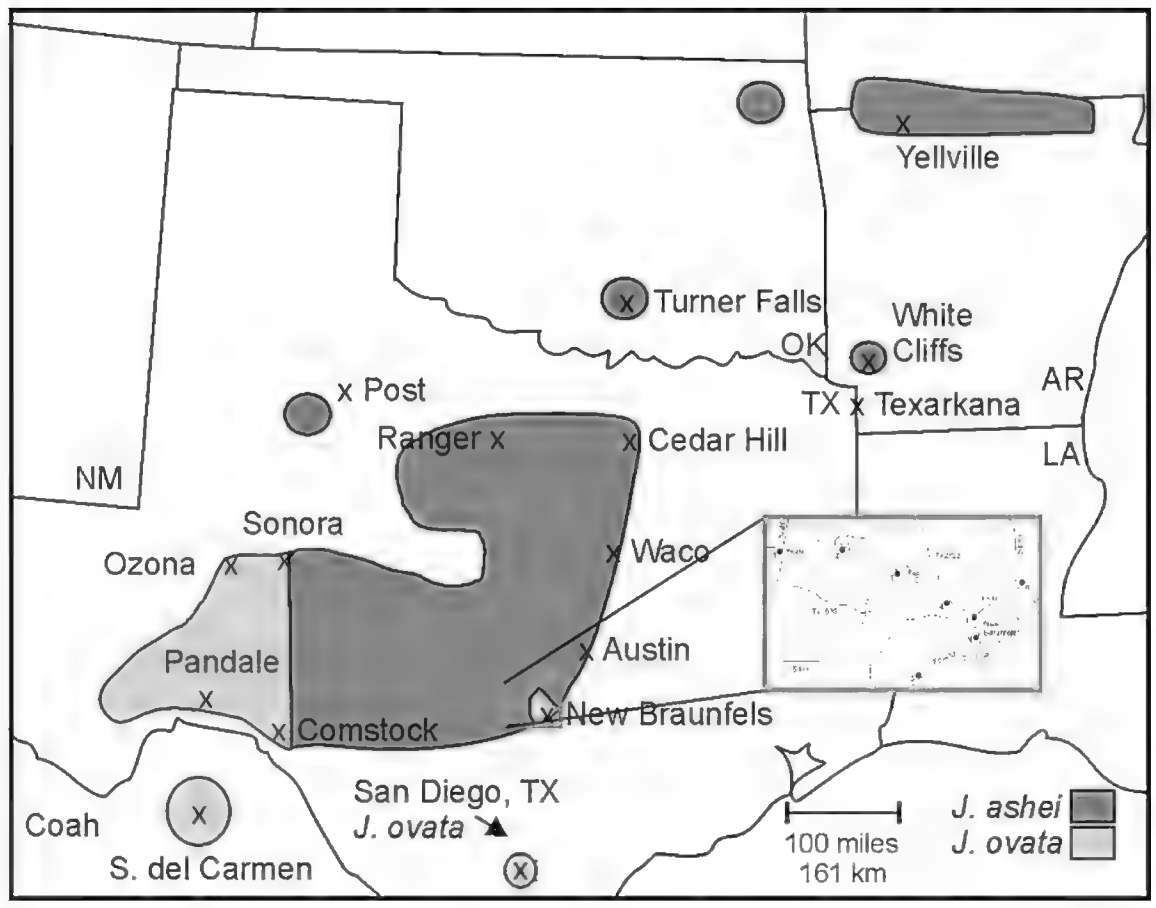

Figure 5. Distribution of *J. ashei and J. ovata.* Adapted from Adams (2014). The detailed Comal Co. study area is indicated by the fly-out box. X's mark the populations studied.

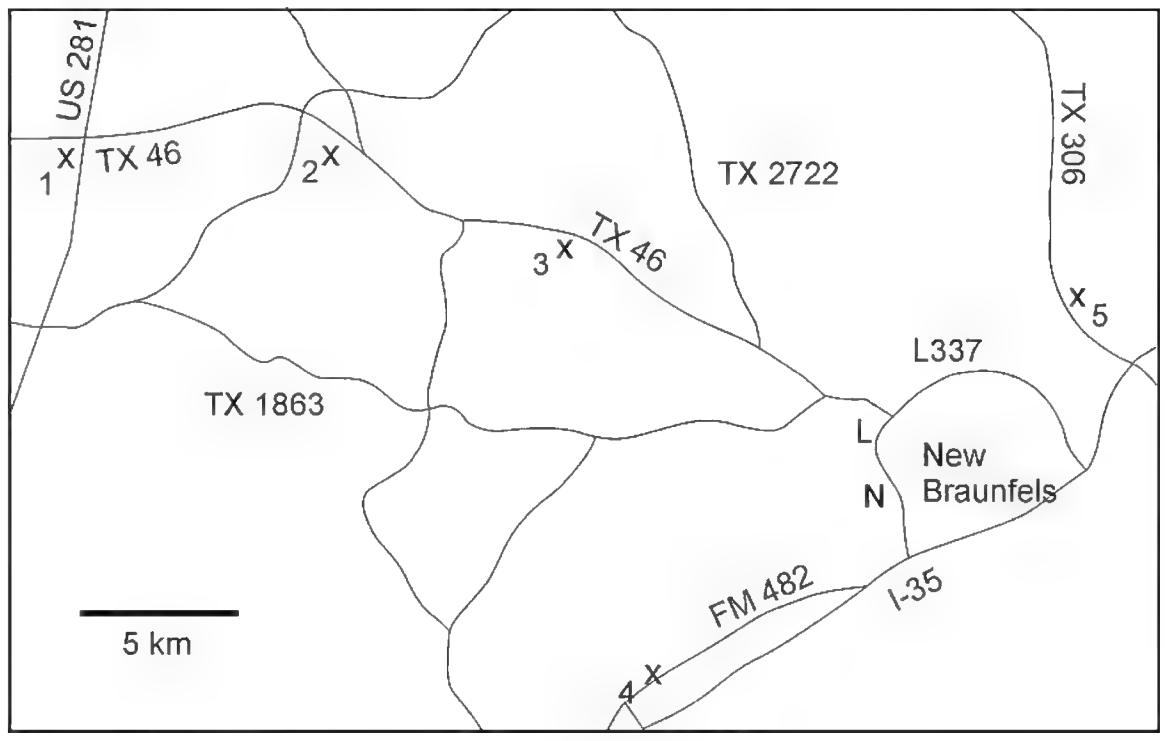

Figure 6. Populations sampled in Comal Co. and New Braunfels area in the fly-out box.

Specimens used in this study:
Juniperus ashei: Comal Co., TX: Popn. 1, jct TX46 & US 281, *Adams 11295, 11296, 11297*; Popn. 2, on TX 46, 8 km e of jct TX 46 and US 281, *Adams 11298, 11299, 11300*; Popn. 3, on TX 46, 16 km e of jct TX 46 and US 281, *Adams 11301,* Popn. 5, on TX 306, 1 km nw of Hunter Rd, *Adams 11322, 11323, 11324.*
Yellville AR: *Adams 10215-10219*; White Cliffs, AR: *Adams 14071-14080*; Turner Falls, OK, *Adams 14094-14100*; Cedar Hill, TX: *Adams 12007-12011*; Benbrook Lake, TX *Adams14091-14092*; Ranger, TX: *Adams 12012-12015*; Cameron Park, Waco, TX: *Adams 14081-14090*; Bosque Blvd and Hwy 6, Waco,

TX: Adams 6746, 6752; Texas A & M Extension Station (TAES), Sonora, TX: *Adams 12250-12269*, as part of a study on deer browsing (Adams et al. 2013);
J. ovata: Comal Co., TX: Popn. L, Loop 337, 1 km s of jct TX 46 and Loop 337, *Adams 11314, 11315, 11316*; Popn. N, 40 m w of jct Cedar Elm St. and Madeline St. on Madeline St. (site of the National Big Tree for *J. ashei*), New Braunfels, *Adams 11309, 11317, 11318*; Popn. 4, 100 m n of jct Hubertus Rd. and FM 482 on FM 482, *Adams 11319, 11320, 11321*.
Ozona, TX: *Adams 7470, 7473,12280-12284*; Comstock, *TX 12270-12274*; Pandale, TX: *Adams 12275-12279*; San Diego, TX, escaped seed from tree(s) planted in San Diego Cemetery: Adams 12532-12533. Voucher specimens are deposited at Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS primers utilized. Two indels (at sites 194 & 802) prevented single pass sequencing of the 1270 bp ITS area, so two internal primers were designed (ITS426for and rev (site 410 in sample 12271(*J. ovata*), ITS426for = CCC GTT GAG ATT CCA TG). The primers for trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences were determined by Chromas 2.31 (Technelysium Pty Ltd.).

RESULTS

Four informative cp gene regions were sequenced to compare *J. ashei* with *J. ovata*. These four cp regions had the following informative SNPs and indels: petN-psbM (798 bp), one SNP and 2 indels; trnD-trnT (684bp), 2 SNPs, one indel; trnL-trnF (701bp), 2 SNPs, no indel; and trnS-trnG (823bp), 2 SNPs and 2 indels. All these 4 cp regions distinguished *J. ashei* from *J. ovata*. However, trnS-trnG (trnSG) contained a large, 133bp indel (present in *J. ashei* and absent in *J. ovata*) that could be easily scored on a 1.5% agarose gel. Thus, this marker could be used for an easy detection of the 'ashei' or 'ovata' cp type. Table 2 contains the summary of the DNA analyses as well as the cp classification for individuals, ordered by location. Because cp are inherited from the male (pollen) in the Cupressaceae section that includes *Juniperus* (Adams, 2019; Adams, Miller and Low 2016), the paternal (pollen) parent can be determined for any hybrids found. Every plant collected as *J. ashei* (green highlight in Table 2), except 11320, *ovata* cp, Table 2) had the *ashei* cp DNA. And, every plant collected as *J. ovata* (red highlight, Table 2) had the *ovata* cp DNA.

Sequencing nrDNA (ITS), yielded 1270 bp, with 4 SNPs (sites 258, 302, 303, 758) and 2 indels (sites 194, 802) that distinguish *J. ashei* and *J. ovata*. About half of the plants collected as *J. ashei* were homozygous at the 4 SNP and 2 indel sites and about half were found to be heterozygous at the 4 SNP and 2 indel sites, implying they are hybrids or backcrossed to *J. ovata* (Table 2). About two-thirds of the trees collected in the trans-Pecos Texas area were homozygous at the 4 SNP and 2 indel sites, but four were hybrids or backcrosses and one was homozygous for *J. ashei* in their nrDNA (ITS)r, but had a J. *ovata* cp type (Table 2).

There is a noticeable trend from New Braunfels (with mostly red, *ovata*) to Austin and Waco, with considerable hybrids and introgressants (IG symbol, Fig. 7), then Cedar Hills and Turner Falls (nearly all green, *ashei*). White Cliffs, AR is nearly half *J. ashei* and half *J. ovata* with 3 'pure' *J. ovata* (red) ITS

plants and 3 introgressants (IG), whereas Yellville, AR has only pure *J. ashei* or hybrid ITS, no introgressants (Fig. 7). No plants in the *J. ashei* range had *J. ovata* chloroplasts and no plants in the *J. ovata* range had *J. ashei* chloroplasts. The *J. ashei* chloroplast plants end abruptly west of Sonora; thence westward, all plants have *J. ovata* chloroplasts, although hybrid ITS, and introgressants are found in Comstock and Ozona, respectively. The Pandale plants are the purest *J. ovata* found in this study.

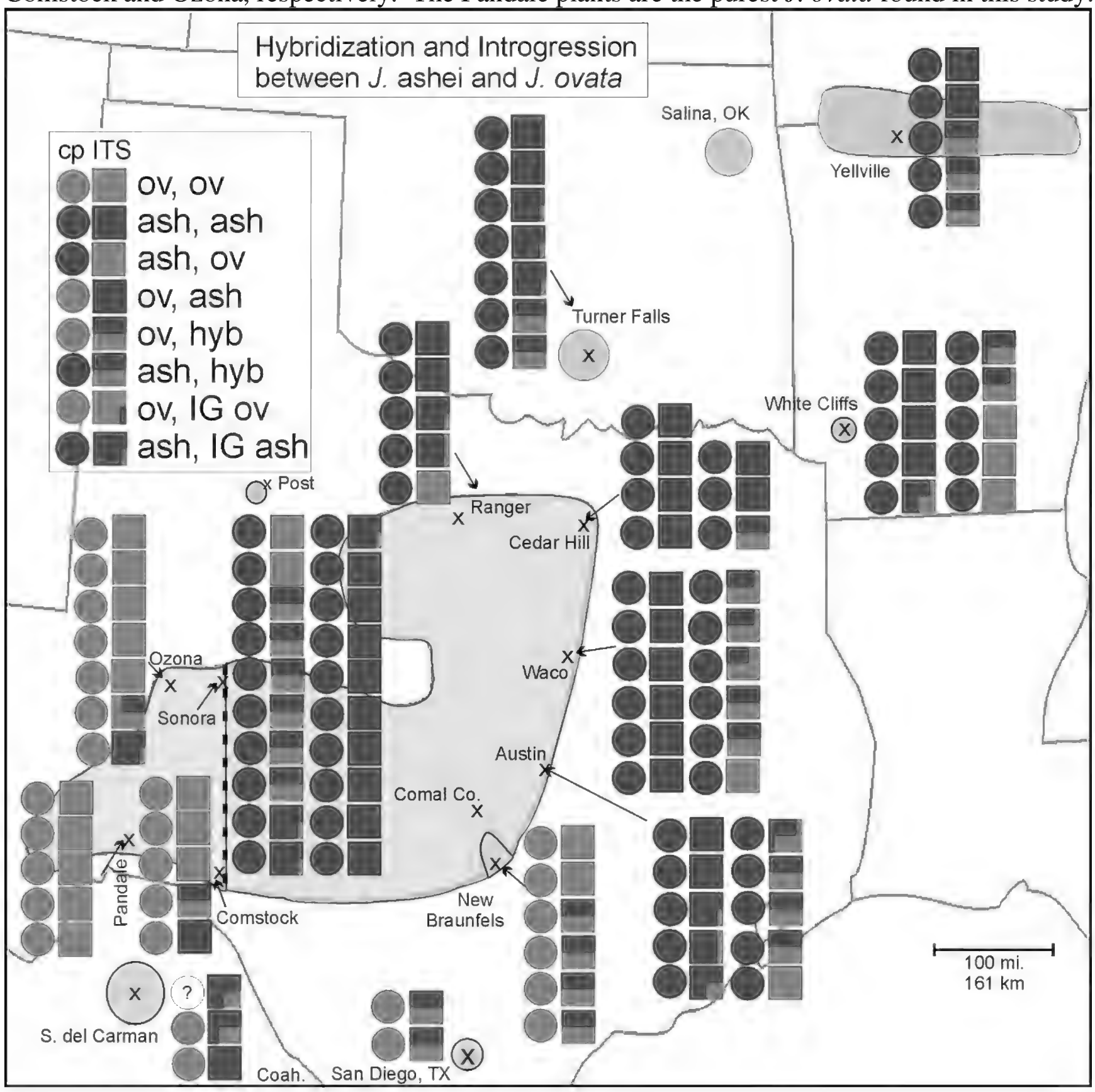

Figure 7. Hybridization and introgression between *J. ashei* and *J. ovata*, in cp DNA and nrDNA (ITS).

The Sonora population also has considerable polymorphic ITS DNA (hybrids and introgressants), but no *ovata* cp DNA. Is there wind in the correct direction to move pollen? Wind data shows that the most wind in January comes from the south and west and north (northerner winds) from Midland to Waco and northward to Oklahoma City and Tulsa (Fig. 8). However, San Antonio has low frequency of wind from the south and Ft. Smith winds are deflected by the Ozark Mtns. east and westward. Because *J. ashei* and *J. ovata* shed pollen in Dec.-Jan-early Feb., it is useful to examine winds from near Sonora (Midland, Fig. 8). This shows the major January winds are from south and west. Clearly copious amounts of *ovata* pollen from the trans-Pecos are near to Sonora. But, none of the ITS hybrids in the Sonora population had *ovata* cp DNA.

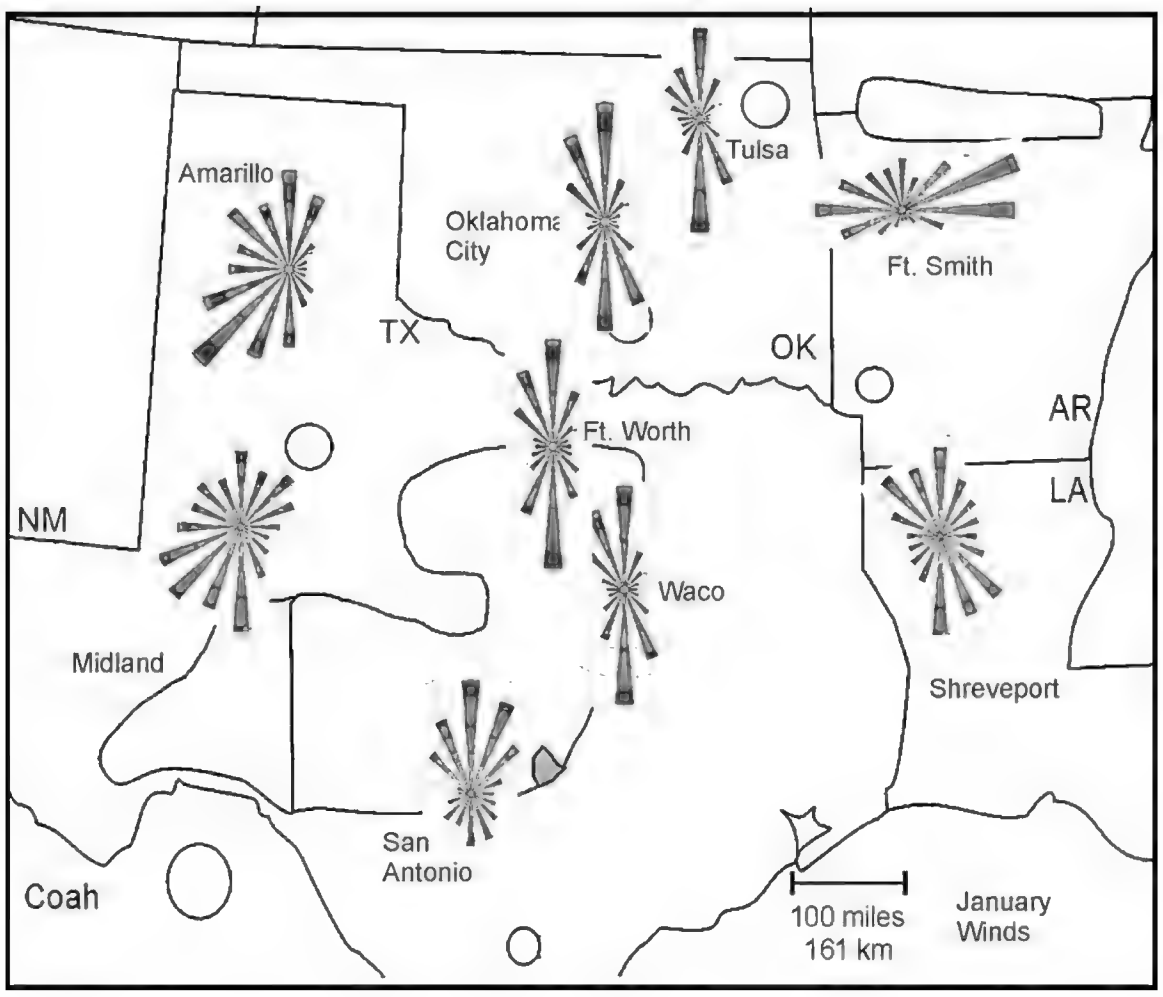

Figure 8. Wind direction (from the arrow outer tips) and velocity for sites in the study area. Velocity normalized, not to scale. Adapted from Wind Rose Resources. wcc.nrcs.duda.gov/climate /windrose.html.

Several other studies in conifers have reported long distance transport (LDT) of pollen from a few km to several hundred km (Szczepanek et al. 2017; Neale and Wheeler 2019; Stewart et al. 2012; Sarvas 1962; Koski 1970; Nichols et al. 1978; Campbell et al. 1999). To effect pollination in distant populations, one needs long distance pollen transport, but also viable pollen when it arrives at a distant population. In fact, several studies have reported that LDT pollen has maintained its viability (Lindgren et al. 1995; Varis et al. 2009; Williams 2010). Pollen from *Juniperus communis*, in the western Alps, was stored at ambient conditions and found to be 40-90% viable for fresh pollen, 20-40% viable after two weeks and 0-10% viable after two months storage (Carmeliello et al. 1990). Finally, it should be mentioned that in a preliminary study on LDT pollen viability, Levetin (Dr. Estelle Levetin, U. Tulsa, per. comm.) found viable *Juniperus* (*J. ashei*) LDT airborne pollen in Tulsa, OK, after having traveled at least 200 mi. because Tulsa has a prevailing south wind in Dec. - Feb. (Fig. 8), the nearest *J. ashei* populations to the south are White Cliffs, AR, Turner Falls, OK or Cedar Hill, TX.

Unfortunately, as attractive as long-distance transport (LDT) of pollen and subsequent fertilization is, it cannot explain the pattern of an absolute lack of *J. ovata* cp in any population of *J. ashei*, nor that none of the F1s have *J. ovata* cp (i.e., obtained by *J. ovata* pollen fertilizing *J. ashei* receptive female cones). Nor are pollen crossing barriers an explanation, because of the existence of F1 hybrids, arising from crosses of pure *J. ovata* with pure *J. ashei*. The lack of *J.* ovata chloroplasts found in the range of *J. ashei*, supports the idea that *J. ovata* genes are introgressed by the movement of F1 hybrid seeds and thence seedlings and eventually, mature F1 hybrid trees.

Birds are well known to eat juniper seed cones ('berries') and widely disperse the seeds (Adams and Thornburg (2010); Phillips 2010; Adams 2014; Holthuijzen, Sharik and Fraser 1987). In fact, cases of junipers endemic to islands are attributed to long distance transport (LDT) by birds. These include *J.*

bermudiana, Bermuda; *J. brevifolia*, Azores, and J. *cedrus*, Canary Islands. Cedar waxwing (*Ampelis cedrorum*) is a major consumer of *J. ashei*, *J. ovata* and *J. virginiana* berries in the winter in central Texas (Phillips, 1910). In fact, Phillips (1910) lists 17 bird species that feed on *J. virginiana* berries. Brugger et al. (1994) researched the winter ranges of cedar waxwings, banded in their summer range in June - August, by recapture in their winter ranges (Dec. - Feb.). It is useful to examine their results concerning banded cedar waxwings in their winter ranges in Texas and Louisiana (Fig. 9). The group included two from WI (Wisconsin), 2 from PA (Pennsylvania), and one each from North (ND) and South Dakota (SD). Their shortest return routes to their summer ranges take the Dakota birds over the Ranger population. The Wisconsin cedar waxwings would fly over Turner Falls, Salina, OK, White Cliffs, AR and the Ozarks - Yellville populations. The Pennsylvania (PA) birds would fly over the White Cliffs, AR and the Ozarks - Yellville populations. It is clear from even this limited study, that there is certainly ample opportunity for cedar waxwings to disperse juniper seeds into all the disjunct population areas, and one should remember that this event has happened every year for thousands of years! So, it is not surprising that *J. ovata* and/or hybrids seeds from New Braunfels, and trans-Pecos Texas have been sown by birds in every conceivable habitat both within Texas and north-northwest of Texas. The same is true for *J. ashei* seeds. It is possible that repeatably some *J. ovata* and/or hybrid seeds fall on suitable sites in the disjunct populations, germinate and grow into reproductive trees, thus injecting germplasm into these '*J. ashei*' populations.

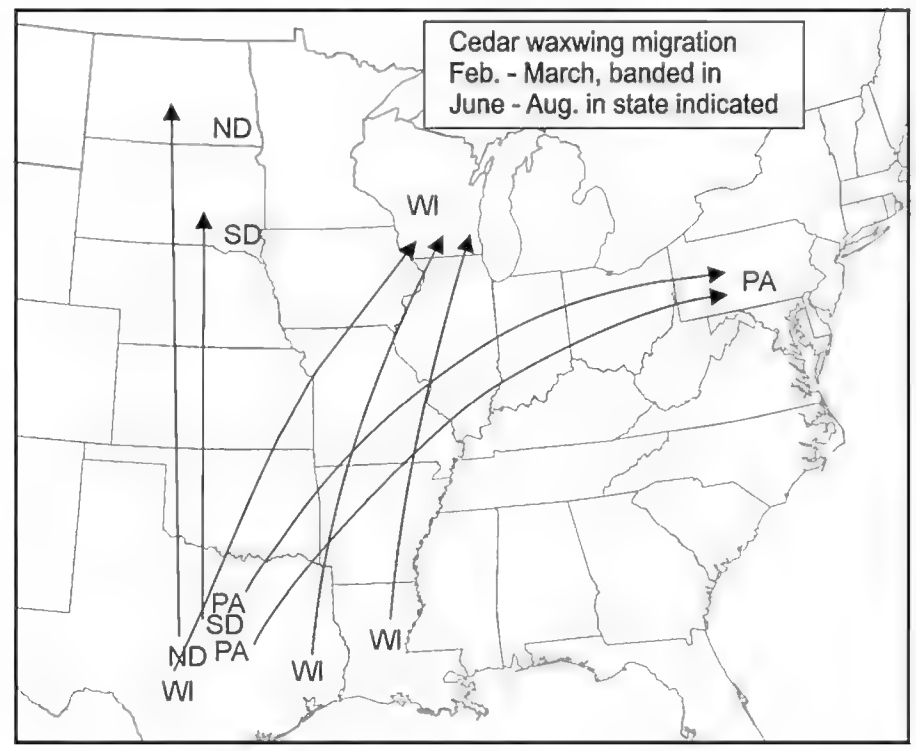

Fig. 9. Cedar waxwing migration in March from their winter home to their summer home states. Adapted from data in Brugger et al. 1994.

It should be noted that the New Braunfels, trans-Pecos, and Sierra del Carman *J. ovata* populations do not appear to be affected by the southern - southwestern migration of birds from ND, SD, WI, PA etc. in the fall, because their summer ranges are outside the distribution of *J. ashei* and *J. ovata*. If birds brought juniper seeds southward, it would most likely be *J. virginiana* and/ or *J. horizontalis*.

The geographic pattern shows no *J. ashei* population examined had only pure *J. ashei* trees (Fig. 7). This is surprising in view of the uniformity found in the terpenoids (Fig. 1). However, terpenoids are well known to be involved as chemical defenses in plants. Seminal papers in the 1970s (Rhoades and Cates, 1976; Cates and Rhoades, 1977; Feeny, 1976) enlightened biologists that plants produce defensive compounds against herbivores. Terpenes and tannins are two types of compounds produced by juniper that are known to deter herbivores (Bernays et al. 1989; Gershenzon and Dudareva 2007). Terpenes can act as feeding deterrents (Gershenzon and Dudareva 2007) and have numerous toxic actions such as central nervous system depression, contact dermatitis, lung function impairment, liver and kidney cysts and even death (Sperling et al., 1967; Savolainen, 1978; Falk et al., 1990) as well as alter microbial fermentation (Schwartz et al. 1980; Nagy et al. 1964). More recently, deer and goats have been shown to selectively browse on *J. ashei* trees that are lower in leaf essential oil concentration (Adams et al. 2014). Woodrats (Neotoma) have also been found to sense the leaf terpenoids to select *J. osteosperma* trees to feed on (Skopec, Adams and Muir, 2019). So, it is very likely that the *J. ashei* (and *J. ovata*) leaf terpenoids are under considerable selection pressure to maintain their chemical defenses. Thus, one might expect wide-spread herbivores, bacteria and fungi to lead to wide-spread terpenoids patterns, as found in *J. ashei* and *J. ovata* (Adams 1977). In contrast, neutral sites such as ITS can show more variation within an area where hybridization occurs. A hybrid plant that has incorporated unusual terpenoid synthase genes from a parent

from a different habitat, into its genome, may be less likely to survive than a tree that has the nuclear genome parts that carry the terpenoid genes from a local parent that has the local array of defensive terpenoids.

Examination of another area of possible hybridization (New Braunfels - Comal Co.) shows that the purest *J. ashei* trees are in the west area near the US 281 and TX 46 junction, whereas the purest *J. ovata* is in or near New Braunfels (Fig. 10). Of particular interest is that only 2 pure *J. ovata* were found in the New Braunfels populations. Population 4 is interesting because both *J. ashei* and *J. ovata* cp parents are present (Fig. 10) in the population. This is the only population found that has both *J. ashei* and *J. ovata* chloroplasts. This population (4) is 'fence row' population: a recent population with plants growing under the barbed wire fence where bird sit to digest juniper female cones and 'plant' the seeds under the fence wire.

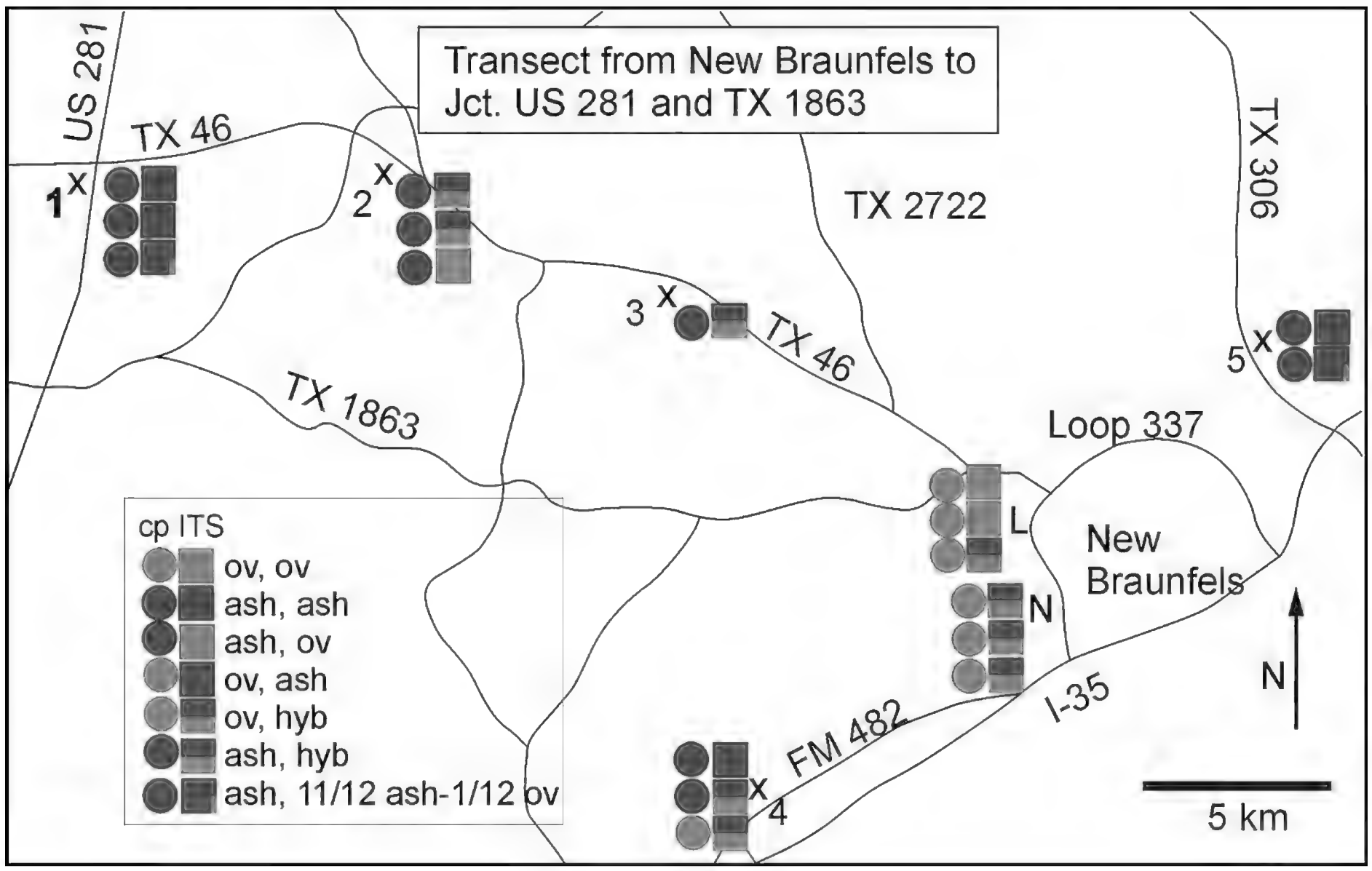

Figure 10. Variation in the New Braunfels - Comal Co. area. Populations 1, 2, 3, differ by 8 km, L = Loop 337/ TX 46 population, N = New Braunfels (city) population.

The two young *J. ovata* trees from near San Diego, TX were growing on a fence-row a few km north of several *J. ovata* trees planted in the San Diego cemetery. The cultivated trees were very likely purchased or dug up from the New Braunfels population due north, rather than the trans-Pecos/ Mexico populations. Both trees were small (2 - 4m) and young. These seem most likely to have been established by birds (cedar waxwings?), that digested the *J. ovata* seed cone fleshy portion and expelled the seeds while sitting on the barbed wire fence. This is a very common dispersal mechanism in junipers and accounts for miles of 'fence-row' junipers in, otherwise, grassland habitats devoid of junipers (Adams 2014). Both plants had *J. ovata* cp, but heterozygous (hybrid) nrDNA (Fig. 7.)

Comparing (using data from the same trees) the terpene classifications (Fig. 4, 5) with the DNA data, both data sets show the purest *J. ovata* in L and N (New Braunfels populations) and purest *J. ashei* in

the western population 1 (Figs. 4, 5, 9). Overall, there is good agreement in the terpenoid pattern and the cp, nrDNA pattern in this small area.

DISCUSSION

The idea that the current patterns are a result of relictual mixing (i.e., hybridization during the Pleistocene) that formed the pattern observed, seems implausible because of the presence of many current F1 hybrid plants rather than later generation introgressants. This pattern can best be explained by recurring F1 hybrid seeds being brought into the northern disjunct populations by birds in recent times.

It is instructive to compare the recent reports of hybridization and introgression in *Juniperus* in the western United States with the present results. Figure 11 shows an interesting case where *J. maritima* comprises very uniform populations in the Pacific Northwest, then bordered by a broad zone of unusual plants with *J. scopulorum* cp and pure *J. maritima* ITS DNA, then an area of *J. scopulorum* with evidence of introgression from *J. maritima*, and finally an area of relatively pure *J. scopulorum*. Notice that one population (WO, Wallowa Mtns., OR, Fig. 11) has a mixture of both chloroplast types. There are only 2 plants with [*maritima* cp/hybrid ITS] (WL, Williams Lake), but there are plants with [*scopulorum* cp/hyb or IG] in populations FH, BU, SS, MO and KU in the *J. scopulorum* range. This is very similar to [ashei cp/hyb or IG] plants in this study (Figs. 7, 10). It may be that bird-transported seeds are important in the *J. maritima- J. scopulorum* case also.

A second example of hybridization between *J. arizonica* and *J. coahuilensis* (Adams 2017) shows (Fig. 12) a zone of hybridization between the species (Hueco Tanks, Quitman Mtns.) with some gene flow in both directions, with a very few F1 hybrids inside the home ranges of *J. arizonica* and *J. coahuilensis*. These hybrid areas appear to be in the overlap areas between the taxa; however, it is notable that only one hybrid ITS plant has *J. coahuilensis* cp DNA (sMF, Fig. 12), a similar pattern as we see in Figure 11 and as well as this present study (Fig. 7).

The third study is of hybridization and introgression between *J. blancoi* and *J. scopulorum* (Adams, et al. 2020). This pattern is somewhat like that of *J. maritima* and *J. scopulorum* (Fig. 11) in having a zone of hybridization and introgression with only *J. scopulorum* cp present. A high frequency of wind from the north in March and April was postulated to have been important in the asymmetric occurrence of *J. scopulorum* cp in plants in north Mexico (Adams, et al. 2020). However, again we see (Fig. 13) plants in the hybrid zone with [*scopulorum* cp/pure *blancoi* ITS]. It should be noted that the taxon in the hybrid zone is *J. blancoi* var. *mucronata* (RP Adams) RP Adams. The taxon is thought to have experienced a *scopulorum* chloroplast capture event (Adams et al. 2020), thus explaining the [*scopulorum* cp/*blancoi* v. *mucronata* ITS] genome.

Two of these examples (Figs. 11, 13) have plants in a hybrid zone with a genotype of (sp. A cp, pure sp. B ITS), which is what we found in this study [*ashei* cp, pure *ovata* ITS: Sonora, Ranger, White Cliffs, Waco, Austin, Comal Co, #2] and [*ovata* cp, pure *ashei* ITS: Comstock, S. del Carman].

In addition, all three of these examples share an interesting aspect: the hybrid areas are located in areas with lower juniper densities. In the *J. maritima - J. scopulorum* study, Eastern Washington and Oregon, and Northern Idaho, and Southeastern British Columbia juniper populations are localized and often very small, separated from adjacent populations by 10s or 100s of miles (Fig. 11). In the *J. arizonica* and *J. coahuilensis* study (Fig. 12), junipers are quite rare in the zone of hybridization (Hueco Tanks, Quitman Mtns.). And, the *J. blancoi* and *J. scopulorum* study, *J. blancoi* var. *mucronata* in northern Mexico has only small, isolated populations (Fig. 13).

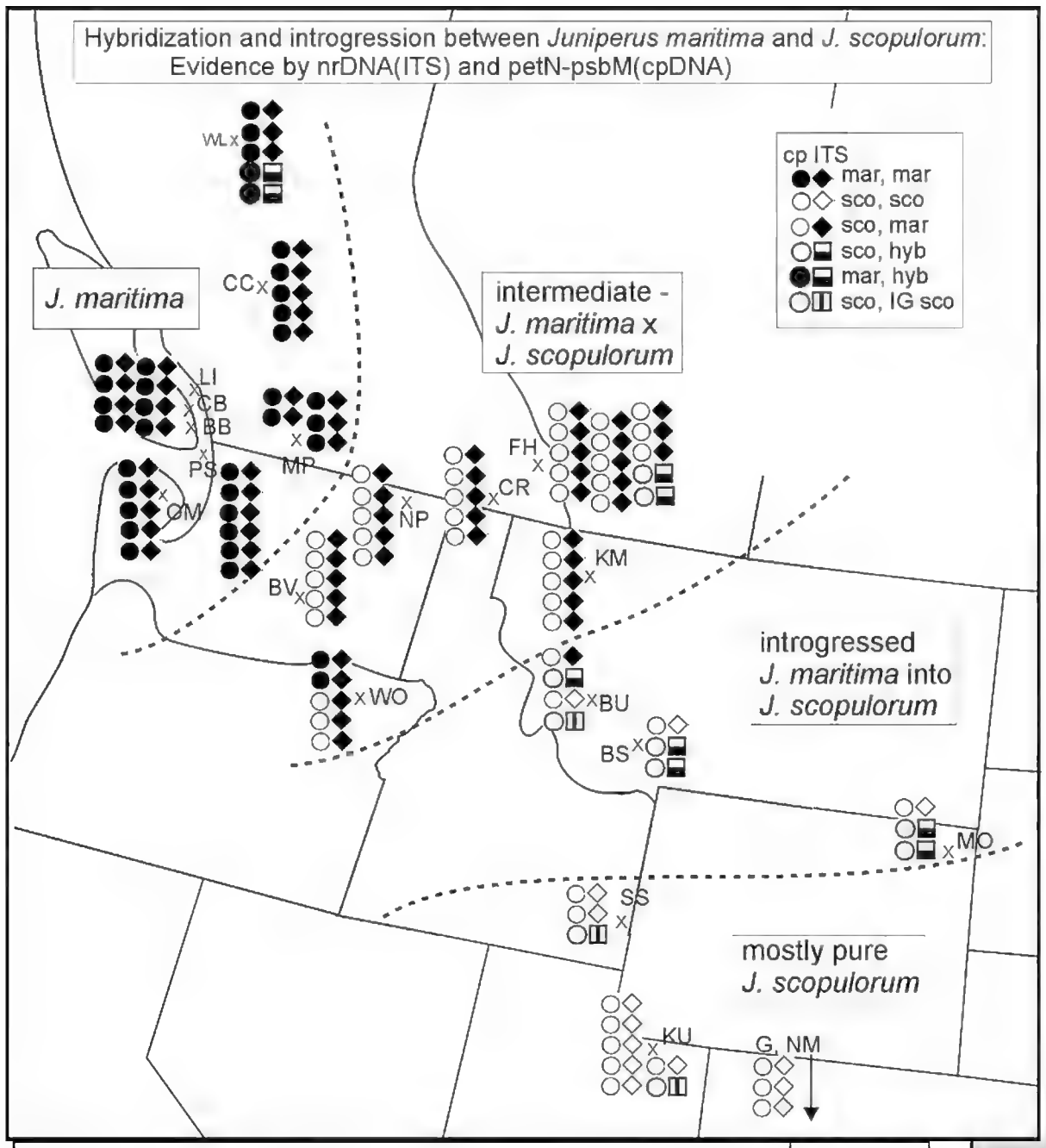

Fig. 11. Hybridization and introgression between *J. maritima* and *J. scopulorum*. Modified from Adams 2015. Note the broad areas of hybridization and introgression. These are areas of low density in these junipers, the hybrids and introgressants.

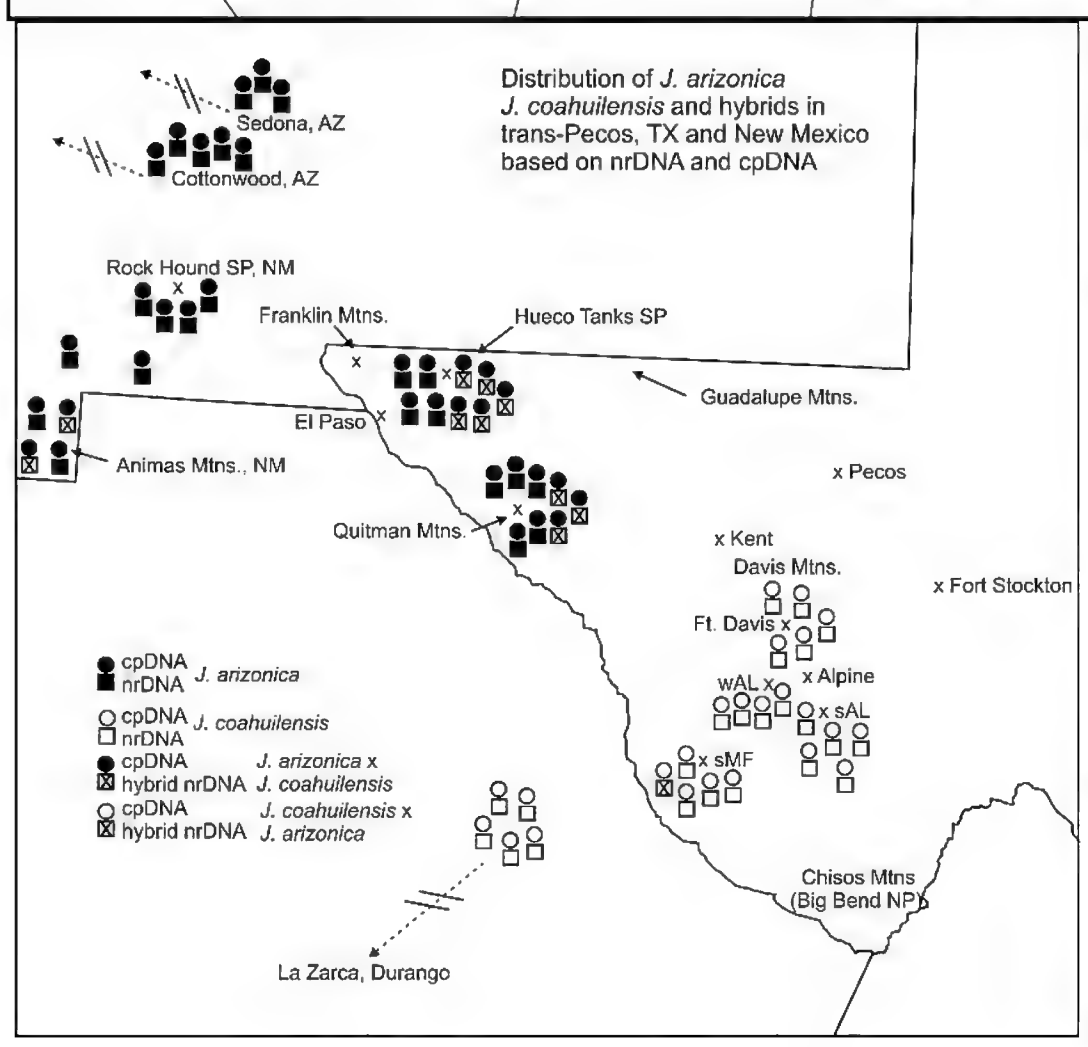

Fig. 12. Hybridization / introgression between *J. arizonica* and *J. coahuilensis* (from Adams 2017). The hybrid zone has a low density of juniper trees.

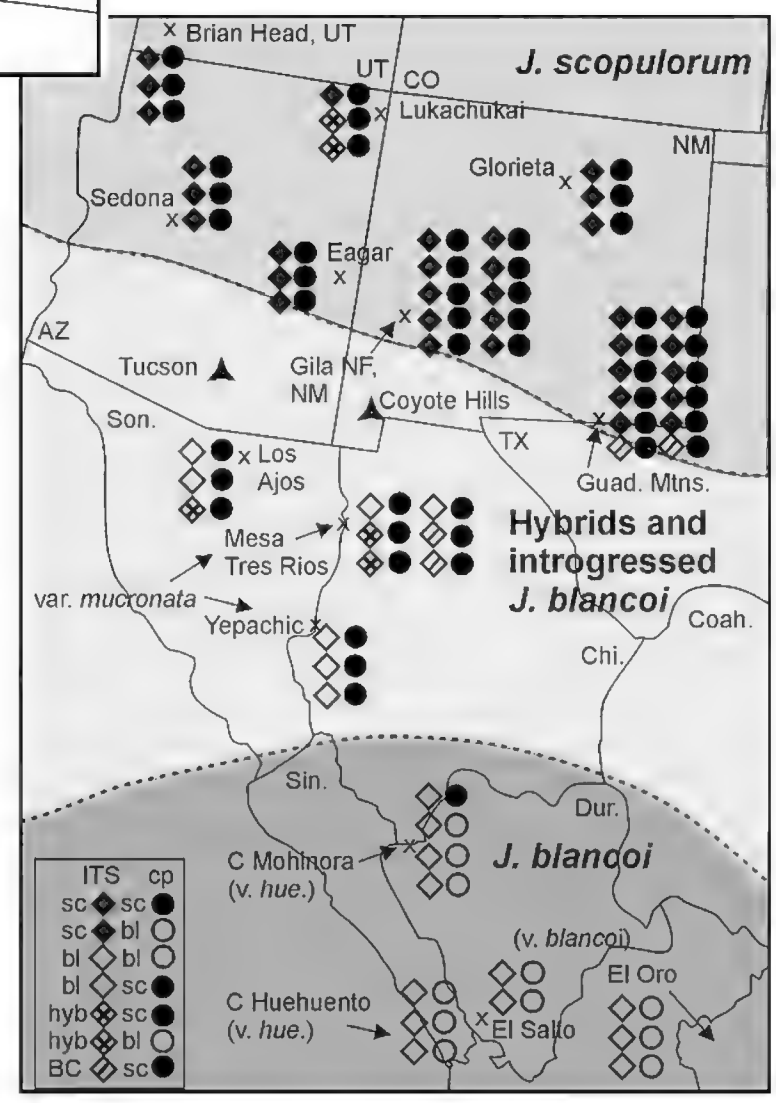

Fig. 13. Hybridization between *J. blancoi* and *J. scopulorum* (from Adams et al. 2020). Red zone has a low density of juniper trees.

In contrast, *J. ashei* is so aggressive that it often forms closed canopy stands of pure *J. ashei* trees in the central Texas limestone hill country and the Arbuckle Mtns, OK. However, the White Cliffs, AR population is very small and mixed in a mesic forest setting. At the Yellville, AR site, *J. ashei* can form cedar glades on limestone outcrops, but these outcrops are often small areas with large gaps between populations.

Friedman and Barrett (2009) reviewed pollination in wind-pollinated plants and consider the leptokurtic distribution of pollen. In *Festuca pratensis* most of its pollen is spread only about 75 m from the source (Rognli et al. 2000). Trees in populations that have been fragmented by disturbances may have pollen dispersal distances of only 65 m (Knapp et al. 2001; Sork et al. 2002). However, although there is good evidence of long-distance transport (LDT) of viable pollen (see above), the presence of copious amounts of local pollen versus limited amounts of LDT pollen would greatly favor pollination by local pollen. Thus, the lack of *J. ovata* cp in hybrids in the Texas Hill country and northern disjunct populations may be just a matter of the overwhelming abundance of local, nearby pollen (of *J. ashei*) compared to the LDT *J. ovata* pollen. It might be noted that all juniper trees in a population normally produce pollen at nearly the same time, so great clouds of pollen are common.

It is interesting to note that Austin, Waco, Sonora, Ranger, and White Cliffs populations each have 1 to 3 trees with perfect *J. ovata* nrDNA (ITS) (Fig. 7). Obviously seeds of this odd combination [*ashei* cp/homozygous *J. ovata* nrDNA] are being produced in the New Braunfels area as one sees one of these plants in population 2 (Fig. 10). A cross of [*ashei* cp/*ovata* ITS] x [*ashei* cp/*ashei* ITS] would yield an ITS hybrid [*ashei* cp/heterozygous ITS]. Birds can easily carry these seeds to the Austin, Waco, Sonora, Ranger, and White Cliffs populations, as well as [*ashei* cp/hybrid ITS] and [*ashei* cp/hybrid ITS] seeds to these and other populations. Holthuijzen and Sharik (1985) reported that juniper seeds that had passed thru the digestive tract of warblers and waxwings germinated at a rate of 55.0% and 27.6% compared to the control rate of 16.1%. It is thought that passage through the digestive tract scarifies the seeds making seeds easier to absorb water and germinate. Johnson (1962) studied the cumulative % germination of *J. monosperma* seeds and found seeds passed through birds germinated sooner (ex. 20%, wk 3, vs. 6% control), but after 10 weeks, both bird and control seeds reached the same level of germination (44%). However, it is very important for a juniper to germinate quickly after rainfall, as the opportunity to establish deep and/ or widespread roots must be done before the moisture is exhausted. This being said, it might be that bird transported seeds, having a long residence time in the digestive tract, may germinate more readily than local seeds and thus have an advantage in the establishment of alien seedlings over indigenous seedlings.

Pleistocene ranges, refugia and re-colonization and the formation of present-day ranges

Although there is considerable evidence of a continuous band of sclerophyllous vegetation from central Texas into northern Mexico during the Tertiary (Axelrod, 1975), it is more productive to focus on events in the Pleistocene, particularly the last Wisconsin and subsequent eras. According to King (1973), the western Ozarks were covered with boreal spruce forest from about 25,000 to at least 13,000 B.P., with pine parkland preceding the boreal spruce forest. The pine parkland and boreal spruce forest both appeared to have been pushed southward from the north (Dillon, 1956).

pre-Wisconsin era:

Based on the uniformity of the current *J. ashei* and *J. ovata* populations (excluding *J. ovata* near New Braunfels), there must have been uniform populations of *J. ashei* on the exposed limestone Edwards Plateau in the pre-Wisconsin era, and likely on the exposed limestone outcrops of White Cliffs, Arbuckle Mtns, and Ozark Mtns. Whether *J. ovata* was wide-spread in the trans-Pecos is not known, but for this discussion we assume it occupied the lower, dryer trans-Pecos and perhaps the northern Chihuahuan desert in Texas, Chihuahua and Coahuila. It seems likely that the current, isolated *J. ovata* population at New Braunfels was not present much more recently.

Wisconsin era vegetation:

Figure 14 shows the hypothetical vegetation during the pluvial period (modified from Adams, 1977). The area south of the Ozarks may have been pine woodland or parkland (see Bryant, 1969). A pine-spruce woodland seems likely on the Llano Estacado of northwest Texas according to Hafsten (1961). Bryant (1969) suggested that, based on pollen profiles, the present Chihuahuan desert area around Del Rio, TX (430 m) was pinyon woodland. Wells (1966), using data obtained from rat middens from the Big Bend region of Texas, concluded that life zones descended about 800 m for pinyon-juniper (*J. pinchotii* in that case), allowing the advance of pinyon-juniper into most of the present desert region between the Big Bend and Del Rio. Typical *J. pinchotii*, and *J. ovata,* have been found growing just south of the Sierra de Carmen

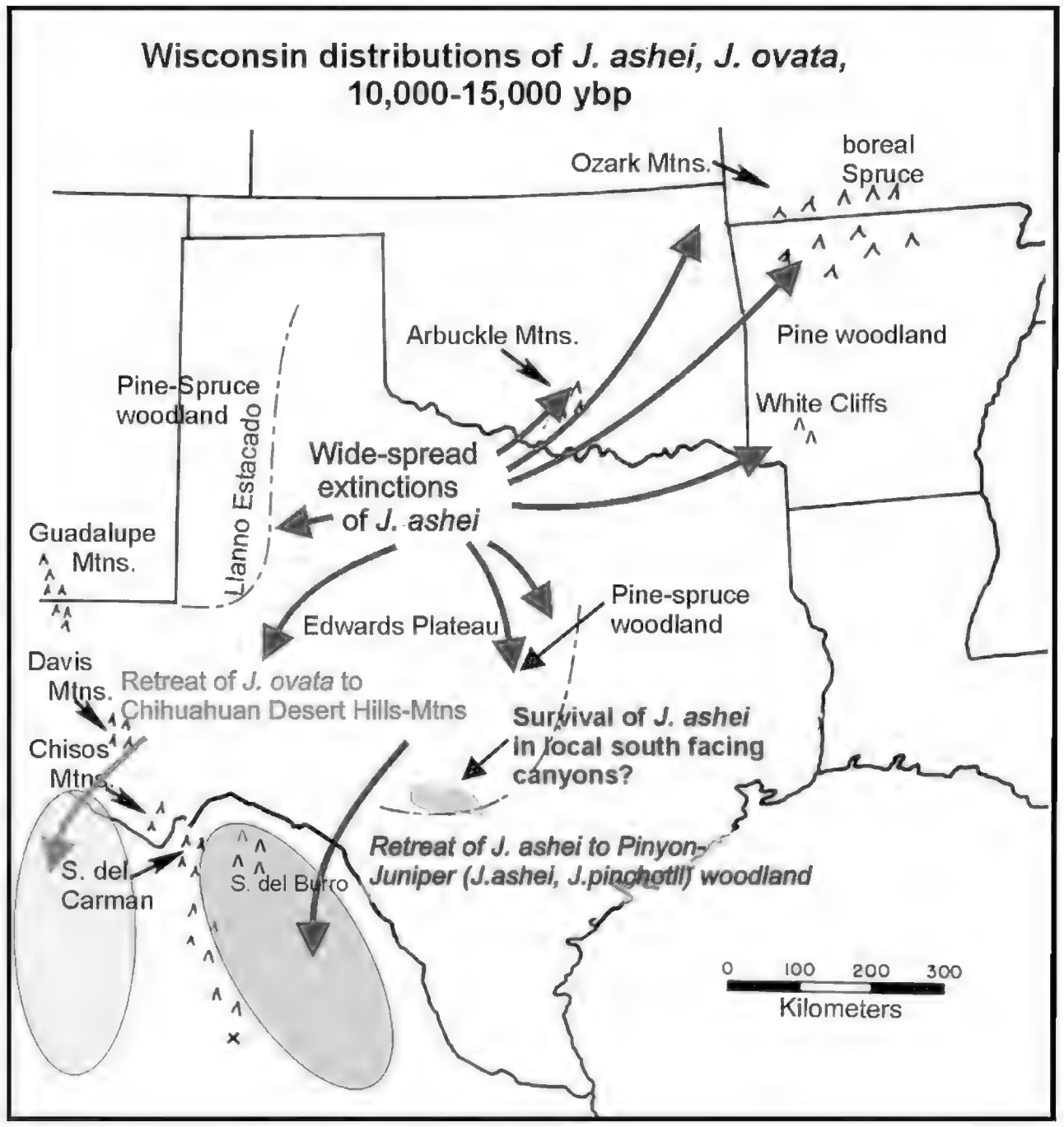

Figure 14. Possible *J. ashei* distribution during the Wisconsin era (from Adams, 1977, 2004).

mountains of the Big Bend region (Adams, 2004). It appears that the Serranias del Burro, Mexico, may have been an important refugium or "island point" in the pinyon-juniper woodland. A mixed deciduous woodland with conifers is postulated in central Texas (Bryant, 1969) based on pollen profiles.

At the end of the Wisconsin glacial advance (10,000 - 13,000 yr bp), the central Texas, Oklahoma and Arkansas populations of *J. ashei* were likely extinct, because this area was a much wetter and cooler spruce woodland (Fig. 14). However, it is possible that some local population(s) of *J. ashei* may have survived on dry, sunny, south facing limestone slopes, especially on the steep south and southeast sides of the Balcones Escarpment. During the cool-wet Wisconsin period, *J. ashei* may have expanded south and west into the Chihuahuan desert (Wells, 1966), but not as far south as Cuatro Cienegas, Coahuila, Mexico (Meyer, 1973). Migration of populations to regions west of the Sierra del Carmen was also possible because *J. ovata* grows at the top of La Cuesta pass just south of the Sierra del Carmen (Adams, 1977). So, it is possible that sympatry of *J. ashei* and *J. ovata* occurred in the Pleistocene. However, if *J. ashei* and *J. ovata* were sympatric in Mexico during the glacial era, and there were hybridization and introgression

between the taxa, a variety of genotypes would have been available including a mixture of *J. ashei*, hybrids, introgressants, and even some 'pure' *J. ovata* for recolonization of central Texas, Arbuckle Mtns., White Cliffs and the Ozarks. However, the presence of only J. ashei pollen throughout the range of *J. ashei* supports the idea that pure *J. ashei* first recolonized central Texas, Oklahoma and the Ozarks. Sometime later *J. ovata* invaded the trans-Pecos area.

Post-glacial (Holocene) re-colonization:

As mentioned above, both *J. ashei* and *J. ovata*, today, have uniform cp within their respective ranges, that supports the idea that the *J. ashei* re-colonization immigrants had uniform *J. ashei* cp. If populations of *J. ashei* were forced to extinction in central Texas, Oklahoma, Arkansas, and Missouri, and subsequent recolonization in the Holocene took place as depicted in figure 15, over a very short time, from a refugium in Mexico (or a relictual population in central Texas) the species may have gone through a selection 'bottleneck' perhaps coupled with genetic drift. The rapid colonization of limestone outcrops (Fig. 15) could then lead to a uniform taxon from central Texas to the Ozarks. Rapid colonization is supported because *J. ashei* has evolved into a very invasive species that, today, is invading disturbed grasslands in the region (Adams et al. 1998; Smith and Rechenthin 1964).

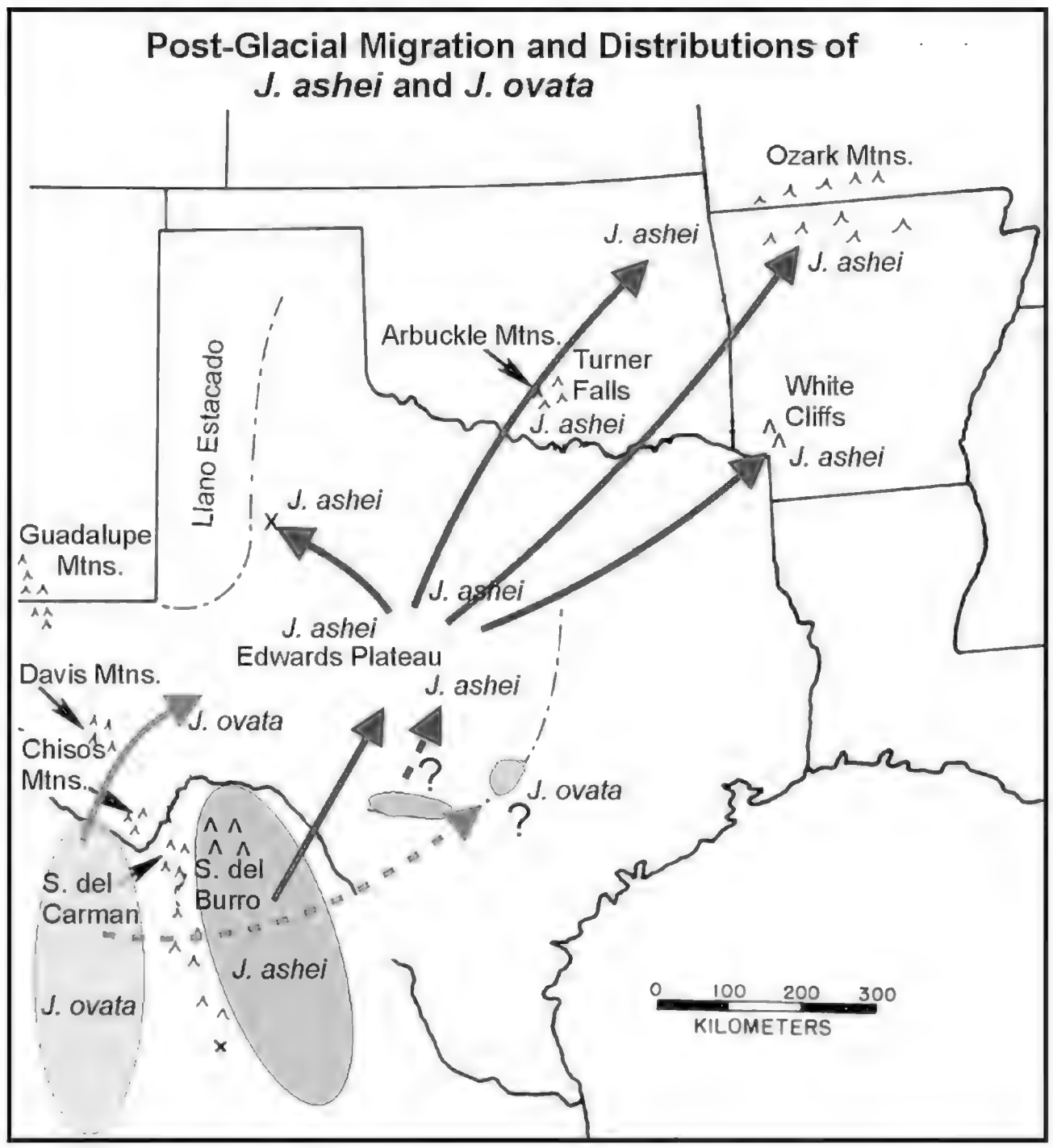

Figure 15. Postulated post-glacial recolonization of *J. ashei* onto limestone producing very uniform populations (Adams, 1977, 2004).

The same argument can be made for uniform ancestral *J. ovata*, that quickly invaded open habitat in the Holocene in the trans-Pecos region. The disjunct, New Braunfels population seems most likely to be a long-distance transport event by birds from the trans-Pecos or northern Mexico *J. ovata* populations. Additional research should resolve some of the un-answered questions in this study.

ACKNOWLEDGEMENTS

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Table 2. Classification of *J. ashei* and *J. ovata* samples based on trnSG and nr ITS DNA. **Orange highlights** are putative hybrid sites. Note that ITS sites 194 and 802 are indel sites scored (when aligned) as: T or - (no T), and A or - (no A), respectively.

coll #	location, field id	trnSG	ITS	194	258	302	303	758	802
10215	Yellville, AR ashei	ashei	ashei	-	C	G	T	A	A
10218	Yellville, AR ashei	ashei	ashei	-	C	G	T	A	A
10216	Yellville, AR ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
10217	Yellville, AR ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
10219	Yellville, AR ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
14071	White Cliffs, AR ashei	ashei	ashei	-	C	G	T	A	A
14076	White Cliffs, AR ashei	ashei	ashei	-	C	G	T	A	A
14079	White Cliffs, AR ashei	ashei	ashei	-	C	G	T	A	A
14080	White Cliffs, AR ashei	ashei	ashei	-	C	G	T	A	A
14072	White Cliffs, AR ashei	ashei	ovata	T	T	A	C	G	-
14073	White Cliffs, AR ashei	ashei	ovata	T	T	A	C	G	-
14074	White Cliffs, AR ashei	ashei	ovata	T	T	A	C	G	-
14075	White Cliffs, AR ashei	ashei	F2 hyb?	T/-	C/T	A	T	A	A
14077	White Cliffs, AR ashei	ashei	hyb BC ovata	T/-	C/T	A	C/T	A/G	A/-
14078	White Cliffs, AR ashei	ashei	hyb BC ashei	T/-	C/T	A/G	C/T	A/G	A
14096	Turner Falls, OK ashei	ashei	ashei	-	C	G	T	A	A
14100	Turner Falls, OK ashei	ashei	ashei	-	C	G	T	A	A
14094	Turner Falls, OK ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
14095	Turner Falls, OK ashei	ashei	ashei BC ovata	-	C	A/G	T	A	A
14097	Turner Falls, OK ashei	ashei	ashei BC ovata	-	C	A/G	T	A	A
14098	Turner Falls, OK ashei	ashei	ashei BC ovata	-	C	A/G	T	A	A
14099	Turner Falls, OK ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12007	Cedar Hill, TX ashei	ashei	ashei	-	C	G	T	A	A
12008	Cedar Hill, TX ashei	ashei	ashei	-	C	G	T	A	A
12010	Cedar Hill, TX ashei	ashei	ashei	-	C	G	T	A	A
12011	Cedar Hill, TX ashei	ashei	ashei	-	C	G	T	A	A
12009	Cedar Hill, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
14091	Benbrook TX ashei	ashei	ashei	-	C	G	T	A	A
14092	Benbrook TX ashei	ashei	ashei	-	C	G	T	A	A
12012	Ranger, TX ashei	ashei	ashei	-	C	G	T	A	A
12016	Ranger, TX ashei	ashei	ashei	-	C	G	T	A	na
12013	Ranger, TX ashei	ashei	ovata	T	T	A	C	G	-
12014	Ranger, TX ashei	ashei	ashei BC ovata	-	C	A	T	A	na
12015	Ranger, TX ashei	ashei	ashei BC ovata	-	C	A/G	T	A	na
14082	Cameron Pk, Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
14084	Cameron Pk, Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
14086	Cameron Pk, Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
14089	Cameron Pk, Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
14090	Cameron Pk, Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
14081	Cameron Pk, Waco, TX ashei	ashei	hyb BC ovata	T/-	C/T	A/G	C/T	G	-
14083	Cameron Pk, Waco, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
14085	Cameron Pk, Waco, TX ashei	ashei	hyb BC ovata	T/-	C/T	A	C/T	A/G	A/-
14087	Cameron Pk, Waco, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
14088	Cameron Pk, Waco, TX ashei	ashei	hyb BC ovata	T/-	C/T	A	C/T	A/G	A/-
6746	Bosque Blvd., Waco, TX ashei	ashei	ashei	-	C	G	T	A	A
6752	Bosque Blvd., Waco, TX ashei	ashei	ovata	T	T	A	C	G	-
12030	West Lake Hills, Austin, TX ashei	ashei	ashei	-	C	G	T	A	A
12031	West Lake Hills, Austin, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12032	West Lake Hills, Austin, TX ashei	ashei	ashei	-	C	G	T	A	A
12033	West Lake Hills, Austin, TX ashei	ashei	ashei BC ovata	-	C/T	A	C/T	A/G	A/-
12034	West Lake Hills, Austin, TX ashei	ashei	ashei BC ovata	-	C/T	A	C/T	A/G	A/-
12035	West Lake Hills, Austin, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12036	West Lake Hills, Austin, TX ashei	ashei	ashei BC ovata	-	C	A/G	T	?	na
12037	West Lake Hills, Austin, TX ashei	ashei	ashei BC ovata	-	C	A	T	A	A
12038	West Lake Hills, Austin, TX ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12039	West Lake Hills, Austin, TX ashei	ashei	ovata	T	T	A	C	G	-
11296	Comal Co, TX ashei T46&281	ashei	ashei	-	C	G	T	A	A
11297	Comal Co, TX ashei T46&281	ashei	ashei	-	C	G	T	A	A
11295	Comal Co, TX ashei T46&281	ashei	ashei BC ovata	-	C	A/G	T	A	A
11298	Comal Co, TX ashei T46,5mi E.	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11299	Comal Co, TX ashei T46,5mi E.	ashei	ovata	T	T	A	C	G	-
11300	Comal Co, TX ashei T46,5mi E.	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-

coll #	location, field id	trnSG	ITS	194	258	302	303	758	802
11301	Comal Co, TX ashei T46 10mi E	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11319	Comal Co, TX ashei Huber. Rd.	ashei	ashei	-	C	G	T	A	A
11320	Comal Co, TX ashei Huber. Rd.	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11321	Comal Co, TX ashei Huber. Rd.	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11323	Comal Co, TX ashei FM306	ashei	ashei	-	C	G	T	na	na
11322	Comal Co, TX ashei FM306	ashei	ashei BC ovata	-	C	A/G	T	A	A
11309	New Braunfels ovata Cedar Elm St.	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11317	New Braunfels ovata Cedar Elm St.	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11318	New Braunfels ovata Cedar Elm St.	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
11314	New Braunfels ovata Loop 337	ovata	ovata	T	T	A	C	G	-
11316	New Braunfels ovata Loop 337	ovata	ovata	T	T	A	C	G	-
11315	New Braunfels ovata Loop 337	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12251	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12256	Sonora, TX TAES ashei	ashei	ashei	-	C	A	T	A	na
12257	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12258	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12259	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12263	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12266	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12268	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12269	Sonora, TX TAES ashei	ashei	ashei	-	C	G	T	A	A
12250	Sonora, TX TAES ashei	ashei	ovata	T	T	A	C	G	na
12252	Sonora, TX TAES ashei	ashei	ovata	T	T	A	C	G	-
12253	Sonora, TX TAES ashei	ashei	ashei BC ovata	T/-	C/T	A/G	T	A	na
12254	Sonora, TX TAES ashei	ashei	ashei BC ovata	-	C	A/G	T	A	na
12255	Sonora, TX TAES ashei	ashei	ashei BC ovata	-	C	A/G	T	A	A
12260	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12261	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12262	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12264	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12265	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12267	Sonora, TX TAES ashei	ashei	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
7470	Ozona, TX ovata holotype tree	ovata	ovata	T	T	A	C	G	-
12281	Ozona, TX ovata	ovata	ovata	T	T	A	C	G	-
12282	Ozona, TX ovata	ovata	ovata	T	T	A	C	G	-
12283	Ozona, TX ovata	ovata	ovata	T	T	A	C	G	-
12284	Ozona, TX ovata	ovata	ovata	T	T	A	C	G	-
7473	Ozona, TX ovata	ovata	ashei BC ovata	-	C	A/G	T	A	A
12280	Ozona, TX ovata	ovata	ovata BC ashei	T/-	C/T	A	C/T	G	A/-
12270	Comstock, TX ovata	ovata	ashei	-	C	G	T	A	A
12271	Comstock, TX ovata	ovata	ovata	T	T	A	C	G	-
12272	Comstock, TX ovata	ovata	ovata	T	T	A	C	G	-
12273	Comstock, TX ovata	ovata	ovata	T	T	A	C	G	-
12274	Comstock, TX ovata	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12275	Pandale, TX ovata	ovata	ovata	T	T	A	C	G	-
12276	Pandale, TX ovata	ovata	ovata	T	T	A	C	G	-
12277	Pandale, TX ovata	ovata	ovata	T	T	A	C	G	-
12278	Pandale, TX ovata	ovata	ovata	T	T	A	C	G	-
12279	Pandale, TX ovata	ovata	ovata	T	T	A	C	G	-
12532	San Diego, TX ovata	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
12533	San Diego, TX ovata	ovata	ashei X ovata	T/-	C/T	A/G	C/T	A/G	A/-
0098	Sierra de. Carman, MX ovata	ovata	ashei BC ovata	-	C/T	A/G	T	A/G	A
0099	Sierra de. Carman, MX ovata	ovata	ashei BC ovata	T/-	C/T	A/G	T	A/G	A
1092	Sierra de. Carman, MX ovata	ovata	ashei	-	C	G	T	A	A

194 indel CCTTT= T insert=ovata; CCTT=del = ashei. 258: xGAATGCC; 302: GAAGAGx; 303: x TCGGAC; 758: xAAGTGCGAT; 802:xAAAAAAACAT 8As in *ashei*, -7As in *ovata*.

Appendix 1. Comparisons of the per cent total oil for the leaf essential oils of *J. ashei* and *J. ovata*. Large differences in concentrations are highlighted in boldface.

KI	Compound	*J. ashei*	*J. ovata*
921	tricyclene	1.3	1.1
933	**α-pinene**	**0.4**	**3.8**
946	camphene	1.6	1.6
969	sabinene	t	0.3
974	β-pinene	t	-
988	**myrcene**	**0.5**	**2.6**
1001	δ-2-carene	t	-
1002	α-phellandrene	t	t
1008	δ-3-carene	t	0.1
1014	α-terpinene	t	t
1020	**p-cymene**	**2.0**	**0.7**
1024	**limonene**	**3.5**	**7.7**
1025	β-phellandrene	t	t
1054	**γ-terpinene**	**0.2**	**0.8**
1067	cis-linalool oxide (furanoid)	t	-
1078	camphenilone	t	-
1084	trans-linalool oxide (furanoid)	0.3	0.4
1086	terpinolene	t	t
1095	**linalool**	**1.4**	**0.4**
1098	**trans-sabinene hydrate**	**0.2**	**-**
1100	isopentyl 2-methyl butanoate	t	-
1112	3-methyl butanoate, 3-methyl-3-butenyl-	t	t
1118	cis-p-menth-2-en-1-ol	t	t
1122	α-campholenal	t	-
1136	**trans-p-menth-2-en-1-ol**	**0.2**	**-**
1141	**camphor**	**69.1**	**53.3**
1145	camphene hydrate	0.3	0.3
1165	borneol	2.2	2.8
1174	terpinen-4-ol	0.3	0.5
1179	p-cymen-8-ol	0.3	0.1
1186	α-terpineol	0.1	t
1204	**verbenone**	**0.1**	**-**
1207	trans-piperitol	0.2	t
1215	**trans-carveol**	**0.7**	**t**
1218	endo-fenchyl acetate	t	-
1226	cis-carveol	t	t
1239	**carvone**	**0.8**	**t**
1249	piperitone	t	-
1273	trans-carvone oxide	t	-
1287	**bornyl acetate**	**6.3**	**15.6**
1289	p-cymen-7-ol	t	-
1298	carvacrol	t	-
1339	trans-carvyl acetate	t	t
1340	piperitenone	t	-
1548	**elemol**	**0.2**	**0.9**
1649	β-eudesmol	t	0.4
1652	α-eudesmol	t	0.5
1968	**sandaracopimara-8(14),15-diene**	**0.2**	**-**
1987	manoyl oxide	3.6	3.2
2055	abietatriene	0.2	0.2
2087	abietadiene	0.2	0.3
2282	sempervirol	1.1	0.5
2314	trans-totarol	0.7	0.3
2331	trans-ferruginol	0.2	0.1

values of 0.05% or less are denoted as traces (t). Unidentified components less than 0.5% are not reported. KI is the arithmetic retention index in DB-5.

Taxonomy and morphology of *Macrochytrium* (Chytridiomycota)

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ABSTRACT

Macrochytrium is a potentially large, monocentric chytrid, and has attracted taxonomic attention by virtue of its size and aspects of morphology. A single species, *M. botrydioides*, was initially described (Minden, 1902)—for many years the only published taxon. Eventually, two additional taxa (one species and one variety) were proposed, both proving to be nomenclaturally invalid. Irrespective of nomenclature, the rather considerable variation described for *M. botrydioides* does not appear to represent distinct, additional taxa. *Macrochytrium* is morphologically interesting in that the sporangium, in most cases, does not arise directly from the apex of the primary thallus-axis, but forms subapically (laterally) from this structure—the original apex often remaining as a small protuberance or bulge below the sporangium. A difficulty in assessing the morphology of *Macrochytrium* is that its earliest stages of development (germination of the zoospore-cyst) remain unknown, leading to difficulties in attempting to assign a particular 'thallus-type' to this genus. The general systematic relationships of *Macrochytrium*, as a member of the Chytridiomycota, seem reasonably clear, based in part on the perceived form and structure of the zoospore. However, its ordinal/familial relationships within Chytridiomycetes remain uncertain, even though the sporangium is distinctly operculate. Knowledge of morphology (especially early development) of this saprotrophic (probably cellulosic) genus—occurring on decaying fruits and twigs—could benefit from further collection, culturing, and life-cycle observations. Ultrastructural and molecular analyses would surely clarify its relationships. *Published on-line www.phytologia.org Phytologia 102(2): 75-82 (June 24, 2020). ISSN 030319430.*

KEY WORDS: Chytrid, cyst, monocentric, operculum, rhizoids, sporangium, thallus, zoospores.

Macrochytrium is not common, but is widely distributed (Europe, including parts of Scandinavia; North America; India). There is a superficial resemblance to the bulbous alga, *Botrydium*; hence the epithet of the species name, *M. botrydioides* (Minden, 1902). Interest in this saprotrophic genus has arisen in part because, in robust examples, it is the largest known monocentric chytrid (Karling, 1977; Das-Gupta and John, 1988), and can be visible to the naked eye. Maximum sporangial lengths of approximately a millimeter have been reported, although sporangia are often smaller than this. It has also proved of interest because of an unusual sporangiate-thallus development (Bessey, 1950; Karling, 1977); ontogeny should be further investigated since earliest developmental stages (zoospore-cyst germination, potential nuclear migration?) remain unknown. In spite of interest in *Macrochytrium*, and informative summaries (e.g., Sparrow, 1960; Karling, 1977), there is no current, detailed taxonomic/nomenclatural review of the genus—this, a goal of our investigation. Considered a member of the Chytridiomycota, relationships within this phylum have continued to be uncertain—though some ideas concerning relationship are more plausible than others (discussed herein). Interestingly, a form similar to *Macrochytrium* is demonstrably ancient; Krings et al. (2016) described fossil forms resembling *Macrochytrium* and *Blastocladiella* from shale or soil-like layers preserved within the 410-Ma-old Rhynie-chert (Scotland); certain of their illustrations indicate the presence of a sporangial operculum, an operculum being suggestive of *Macrochytrium* not *Blastocladiella*.

TAXONOMIC HISTORY OF GENUS *MACROCHYTRIUM*

Minden (1916) provided an extensive description and discussion of *Macrochytrium*, including a detailed diagnosis of this genus and, following, a diagnosis of species *M. botrydioides*. Minden (1916) clearly indicated these taxa as being, respectively, a new genus and new (the only) species. However, Minden (1911), in more summary form, had already published these names, with descriptive material and illustrations. Even more relevant to nomenclature is that Minden (1902) *first* published the name *Macrochytrium botrydioides* in a still-earlier publication, with descriptive information of the thallus, sporangium, and limited but critical information on the zoospore. Thus, the original publication of *Macrochytrium botrydioides* should be accepted (as it has usually been) as 1902, not 1911 or 1916. There is no doubt that Minden (1902) was discussing a new genus and species (viz., use of the name *Macrochytrium botrydioides*, with descriptive information). This 'combined' description, in fact, serves to validate both genus and species (cf. Article 38.5, 38.6 of ICNAFP).

Macrochytrium continued to be monotypic (cf. Sparrow, 1960; Karling, 1977), containing only M. *botrydioides* (misspelled '*botryoides*' in Clements and Shear, 1931), until Das-Gupta (1982) listed a 'new species' *Macrochytrium botrydiella*—this name not accompanied by a description—thus a *nomen nudum* (Articles 38.1, 38.2, 50B and Glossary, ICNAFP) and therefore illegitimate (Art. 6.5). From the name '*M. botrydiella*,' one could speculate Das-Gupta may have observed a small specimen(s) of *M. botrydioides*. Subsequently, Das-Gupta and John (1988) indeed described a small form of *Macrochytrium botrydioides*—as a new variety, *M. botrydioides* var. *minutum*. In addition to smaller size, they considered aspects of its morphology and development (later here discussed) to not be identical to typical *M. botrydioides*. Although a description of this 'new variety' was given (Das-Gupta and John, 1988), no Latin diagnosis was provided (required at the time; ICNAFP, Article 39.1). Thus, this varietal name is not valid. Das-Gupta and John (1988) gave no indication if this 'variety' was comparable to the 'species' Das-Gupta proposed in 1982; both names are accounted for in Longcore (1996); only the variety is listed in *Index Fungorum* (in addition to 'typical' *M. botrydioides*). No other names have been introduced, and *Macrochytrium* remains (nomenclaturally) monotypic.

HOW MANY 'TAXA' SHOULD BE RECOGNZED IN THE GENUS?

Regardless of nomenclatural irregularities, invalid names, etc., *should* a taxon (perhaps better said, 'biological entity') additional to *Macrochytrium botrydioides* be recognized?—i.e., based on smaller size (and associated morphology). This question was addressed (see below) by Johnson (1968) regarding a collection (Lake Itasca, Minnesota) of small specimens of *Macrochytrium* (maximum sporangial length = 46 μm). Sporangial sizes (of ca. 40 μm) reported by Das-Gupta and John (1988) are similar, but they did not mention Johnson's work in this regard. Minden (1911) reported sporangial lengths up to 900 μm (enormous, among monocentric chytrids). A range of sporangial lengths of 300-800 μm was subsequently noted (Minden, 1916)—even 300 μm is very large for chytridiomycetous organisms. Lund (1934) reported sporangial lengths of 45-284 μm, thus of apparent intermediate size. Lund's account is confusing, since he reported a 'plant-length' (including sporangium, "basal cell," and rhizoidal axis?) of 100-558 μm. It is difficult to tell, in some publications, how much of the actual sporangium was measured, since a transverse wall often comes to delimit a smallish, lower (non-fertile) portion—only somewhat distinct from the thallus-branch axis bearing it (Minden, 1916, figs. 79, 82).

Johnson (1968) concluded there was insufficient basis for describing a new taxon based on smaller size, and one questionable morphological difference (later discussed); we tentatively agree, though the matter is not resolved. Morphological differences observed by Das-Gupta and John (1988) in their smallish specimens are perhaps explainable (they allowed as an 'alternative explanation' to taxonomic differences) by the perhaps young stages of sporangiate-thalli available to them; yet, they favored the idea that reliable taxonomic differences were present in this 'small variety'—hence, its formal

description. Morphological matters are further discussed in the following section. We, presently, consider *Macrochytrium* monotypic (without clearly separable entities). Nonetheless, if a smaller variant of *M. botrydioides* (Johnson, 1968; Das-Gupta and John, 1988) is found to exhibit reliable morphological differences, associated with distinctly smaller size, then 'var. *minutum*' (Das-Gupta and John, 1988) could be validated by an additional diagnosis—or, a new variety or species proposed.

MORPHOLOGY AND DEVELOPMENT (See Figures 1-4)

Though gaps in knowledge remain, the morphology and development of *Macrochytrium botrydioides* was described in detail in three publications by Minden (1902, 1911, 1916). Minden's work on *Macrochytrium*—particularly that of 1916—was succinctly summarized (and illustrated) by Gäumann and Dodge (1928) and Gwynne-Vaughan and Barnes (1937), among others. Relatively early-on, thus, *Macrochytrium* was a well-known and carefully (if incompletely) studied organism among researchers investigating 'phycomycetes' (former category for 'algal-like fungi').

In typical morphology, the thallus of *M. botrydioides* consists of a substantial cylindrical axis (deriving *ultimately* from the zoospore, early details lacking) which develops stout, sometimes coarse, rhizoids at the base. Just below the apex of the main axis, a lateral branch typically forms which swells into a clavate structure, soon becoming dominant, in size and position, over the true apex—the latter, in effect, "pushed aside" (Gäumann and Dodge, 1928, p. 45). The dominating lateral-branch continues growth, forming a (potentially large) sporangium. The original apical-axis grows little more (if any) after sporangial establishment, usually remaining as a protuberance below the sporangial base on one side. The spheroidal to ellipsoidal (sometimes obpyriform or almost cylindrical) sporangium continues enlarging, and often eventually becomes delimited (by a transverse wall), toward its base, from a lower sterile portion of the sporangium more or less continuous with the thallus-branch axis. A large mass of smallish zoospores (2.5-3.5 μm each) is formed within the (larger) fertile portion of the sporangium. The sporangium develops a sizeable operculum ('lid') on its apex. Zoospores are released (after opercular dehiscence), often first as a mass surrounded by a membrane (vesicle), the upper part of this mass pressing out through the single pore (where the operculum was). In *Macrochytrium*, the emergence of zoospores within a vesicle seems similar to that of species of *Chytriomyces* (cf. Karling, 1977, p. 130); but a vesicle may not always be present in *Macrochytrium* (Johnson, 1968). However, when a vesicle ruptures, the (posteriorly uniflagellate) zoospores swim individually (as typical chytrid zoospores). When zoospores settle on a substrate (rotting fruit, decaying twigs) they are capable of amoeboid movement (a pliable, more elongate shape) over the substrate-surface; the amoeboid ability of zoospores (which can also occur at an earlier stage) is advantageous (Gäumann and Dodge, 1928) if a substrate-surface is populated by other 'phycomycetes' or bacterial growth. Reports of resting-spores are questionable (Karling, 1977)—probably representing thick-walled sporangia.

Any consideration of the development and life-cycle in *Macrochytrium* should be tempered by admission that the earliest stages of thallus-development in this genus—i.e., details of the germination of the zoospore-cyst—have still not been observed. If it is not known, for example, whether the cyst simply enlarges (elongates) to become the young thallus (and subsequently the sporangium) or whether a germ-tube is formed to accomplish this—and whether the nucleus of the cyst migrates, or remains in the cyst—it is then difficult to place pursuant development in context. Critical is the ability (or maybe better said, inability) to ascertain where remnants (whether readily visible or not) of the zoospore-cyst wind up (i.e., which eventual structure subsumes or perhaps still bears evidence of the original cyst). The fate of the 'upper' cyst-wall can be significant in interpreting the location of the original apex of the thallus. It is not pertinent here to review all developmental thallus-types of Chytridiomycetes; such discussions are found in Whiffen (1944), Roane and Paterson (1974), and Blackwell et al. (2006).

Directly pertinent though is Whiffen's (1944) conclusion, regarding *Macrochytrium*, that [lacking knowledge of earliest developmental stages, i.e., zoospore-cyst germination] it was impossible to be certain of its type of development (she thus omitted the genus from her classification of thallus-types). Incomplete knowledge of thallus-development has indeed hampered systematic understanding of *Macrochytrium*. However, useful comments are possible. The youngest stages actually observed are of the relatively young thallus, which has become elongate and slightly clavate ('baseball-bat' shaped), cf. Karling (1977, p. 277, his figs. 7, 8). The form of this young thallus is unusual; but in the next stage, something more unusual may be observed—in that a lateral (subapical) bud occurs, just below (behind) the thallus-apex; this lateral branch produces the sporangium, and leaves the thallus-apex behind as a bump- or knee-like process (not developing appreciably further). Though unusual among chytrids, this pattern is perhaps not unique. In genus *Scherffeliomyces* Sparrow, the zoospore-cyst (and a portion of the discharge-tube) persists apically on the thallus, as a vestige—the sporangium (and its discharge-papilla) developing, at an angle, subapically below (see Johns, 1956). But, without early developmental stages of *Macrochytrium*, one cannot be confident these developments are truly comparable.

Then, there is the matter of phenotypic plasticity in chytrids (Miller, 1968). There can be variation within species regarding: sporangial shape, presence of an apophysis, extent of rhizoidal system, etc. *Macrochytrium*, indeed, exhibits variation. The tremendous range in sporangial size (even shape), especially the occurrence of small sporangia, may have to do with degree of crowding on a substrate (cf. Johnson, 1968) or age of specimens (small sporangia more common in young specimens; cf. Das-Gupta and John, 1988). There is even variation in developmental pattern; Das-Gupta and John did not observe a lateral-branch displacing the young thallus-apex; apparently, the thallus-apex was observed to produce the sporangium; Johnson (1968), though, did observe evidence of a lateral-branch forming the sporangium, i.e., a remnant where the original thallus-apex terminated. Additionally, Johnson observed a circumferential 'flange' at the base of the fertile sporangium (not related to the 'apical remnant'); this small 'collar' was not evident in illustrations by Das-Gupta and John, but there is a hint of such in illustrations by Lund (1934). Das-Gupta and John did not observe a transverse-wall toward the sporangial base; this was though seen in Johnson's specimens. Again, some observations (e.g., presence of this transverse-wall) may relate to specimen age, not 'taxonomic difference.'

SYSTEMATICS: POTENTIAL RELATIONSHIPS OF *MACROCHYTRIUM*

As indicated above, earliest stages of development remain unknown in *Macrochytrium*. However, life-cycle stages that are known, including the apparent form and structure of the zoospore, suggest this genus should (as most have concluded) be placed in the Chytridiomycota, as opposed to other fungal or 'pseudofungal' phyla, and suggest that some variation observed in the genus is age- or environment-related—arguing, for now, against recognition of taxa additional to the original species, *M. botrydioides*. Familial (even ordinal) relationships of *Macrochytrium* are unclear; a review of suggested relationships is useful, though, some suggestions being more instructive than others.

Minden (1902, p. 824) considered *Macrochytrium* to have "*Chytridineencharakter*" (chytridiaceous features), its relationships thus sought among this general group. Within "Reihe: Chytridiineae," Minden (1911) classified *Macrochytrium* in family Hyphochytriaceae; this presumed familial relationship—based on perceptions of general morphology—was echoed by Gäumann and Dodge (1928) and Gwynne-Vaughan and Barnes (1937). Minden's choice of putative relationship of *Macrochytrium botrydiodes* was, doubtless, based specifically (at least in part) on the single flagellum of the zoospore. Whereas it is true that zoospores of members of the Hyphochytriaceae have a single flagellum, the flagellum in this family is anterior, and of the tinsel type (having lateral, tubular 'hairs'). The single flagellum of the zoospore of *Macrochytrium* is now known to be posterior, and of the whiplash (non-tinseled) type—hence, 'chytridiaceous.' As knowledge of structure and ultrastructure of such 'chytrid-like' (more broadly, 'phycomycetous') organisms progressed, it became clear that

Hyphochytriomycetes are unrelated to Chytridiomycetes—or to true fungi in general—and are related instead to 'pseudofungi,' i.e., 'straminipilous fungi' (see Fuller, 1989; Blackwell and Powell, 2000; Kendrick, 2000; Dick, 2001 and Blackwell, 2009 for changing concepts of 'hyphochytrids'). Because of incomplete data available, lack of clear understanding of distinctions among groups of the catch-all category 'phycomycetes' led some authors (e.g., Wolf and Wolf, 1947) to suggest (on general appearance) a link between certain Chytridiales and the Leptomitales [forms such as *Rhipidium* and *Mindeniella*, with which *Macrochytrium* is sometimes found associated in nature—growing on rotting fruit or twigs]. The Leptomitales, however, have been recognized for some time (see Alexopoulos, 1962) to belong to the Oomycetes, which are unrelated to Chytridiomycetes, but belong (as do hyphochytrids) to group Straminipila (Dick, 2001). A resemblance of *Macrochytrium*, in form and development, to *Blastocladiella* was also suggested (Sparrow, 1943, 1960); indeed, superficial resemblance of these genera can be striking. However, zoospore-structure of the Blastocladiomycota (James et al., 2014; Powell, 2016) is distinct from Chytridiomycota; *Macrochytrium* appears to have chytridiaceous zoospores; also, no members of Blastocladiomycota (known) have *operculate* sporangia (Karling, 1977, dismissed the idea of relationship of *Macrochytrium* to *Blastocladiella*). Members of Blastocladiaceae exhibit bipolar development of the zoospore-cyst (Powell, 2016), whereas chytrids have unipolar development. Since earliest stages of development of *Macrochytrium* are unknown, one cannot be certain it has unipolar development; but this can be inferred, since it is unlikely a sporangial septum would develop *belatedly* (or not at all) if *Macrochytrium* possessed bipolar development; relatedly, the rhizoidal system of *Macrochytrium* forms from thallus already present beneath the developing sporangium, not from a distinct basal-cell as in many Blastocladiomycetes.

Fitzpatrick (1930) suggested that *Macrochytrium* (except for being larger and coarser) resembled the genus *Rhizophydium* (Rhizophydiaceae, Chytridales). *Rhizophydium* is a true chytrid, with a life-cycle and probable development similar to *Macrochytrium*; however, in addition to looking little like *Macrochytrium*, the sporangium of *Rhizophydium* does not possess an operculum (present in *Macrochytrium*), this structure being a major distinguishing character within Chytridiomycetes (Sparrow, 1960). And, the usually delicate rhizoids of *Rhizophydium*, in effect, originate directly from the base of the entirely sporogenous, often delicate, sporangium. Perhaps confusingly, Cox (1939) and Wolf and Wolf (1947) hinted at relationship of *Macrochytrium* to the chytrid family Rhizidiaceae (not the Rhizophydiaceae), possibly based in some genera of this family (e.g., *Siphonaria*) on the occurrence of a single thallus-axis giving rise to the sporangium. There is some similarity of *Macrochytrium* to *Siphonaria*; but, in *Siphonaria* the thallus is truly interbiotic, the sporangium inoperculate, zoospore-discharge can be other than apical, and there is no walled-off 'lower-part' to the sporangium.

Classification of *Macrochytrium* has, thus, been uncertain. Sparrow (1943) placed this operculate genus in order Chytridiales, family Chytridiaceae; however, it was distinct enough that he accorded it is own subfamily, Macrochytrioideae, containing no other taxa (it had earlier been placed in its own family, Macrochytriaceae; Lund, 1934). Sparrow (1960) placed *Macrochytrium* in subfamily Chytridioideae, next to genus *Karlingiomyces*—to which it bears little resemblance. Blackwell et al. (2004) challenged the inclusiveness and relationships of *Karlingiomyces*, considering a possible connection to the '*Lacustromyces* clade' (Longcore, 1993; James et al., 2000). *Karlingiomyces* (*pro parte*) was placed by Longcore and Simmons (2012) in a new, predominantly chitinophilic, chytrid order, Polychytriales (including *Lacustromyces hiemalis*, *Polychytrium aggregatum* and *Neokarlingia chitinophila*)—a further indication that *Karlingiomyces* is not closely related to *Macrochytrium*.

Sparrow (1973) retained *Macrochytrium* in subfamily Chytridioideae (family Chytridiaceae, order Chytridiales), not indicating other possible relationships. Bessey (1950) and Karling (1977), on the other hand, suggested a possible relationship of *Macrochytrium* to the family Entophlyctaceae (Chytridiales); this suggestion at first seemed unlikely because of endobiotic thallus development within this family; however, the genus *Endochytrium* (of family Entophlyctaceae)—based on rhizoidal system, form of the

sporangium, and release of many, small zoospores (contained initially in a membrane)—is indeed a plausible connection. Critical in establishing this relationship would be the observation of early development-stages of *Macrochytrium* to assess potential similarity to *Endochytrium*. *Endochytrium* was eventually determined to belong to the order Cladochytriales (Mozley-Standridge et al., 2009). Intriguing, as well, is genus *Cylindrochytridium*, which Karling (1977) classified near *Endochytrium*. *Cylindrochytridium* has since also been determined to belong to the Cladochytriales (Steiger et al., 2011). The presumed mode of thallus development in *Macrochytrium* resembles that of *Cylindrochytridium* (the similarity of *Macrochytrium* and *Cylindrochytrium* was initially pointed out by Whiffen, 1944). This similarity [in some ways striking] between *Macrochytrium* and *Cylindrochytridium* has even caused confusion; in Crasemann's (1954) detailed physiological study, involving a presumed *Macrochytrium* isolate, she was in fact dealing with a culture of *Cylindrochytridium* (cf. Sparrow, 1960). In spite of informed guesses as to relationship, systematic placement of *Macrochytrium* remains undetermined (cf. Karling, 1977). Molecular studies could resolve taxonomic relationships, and morphological studies could clarify early development.

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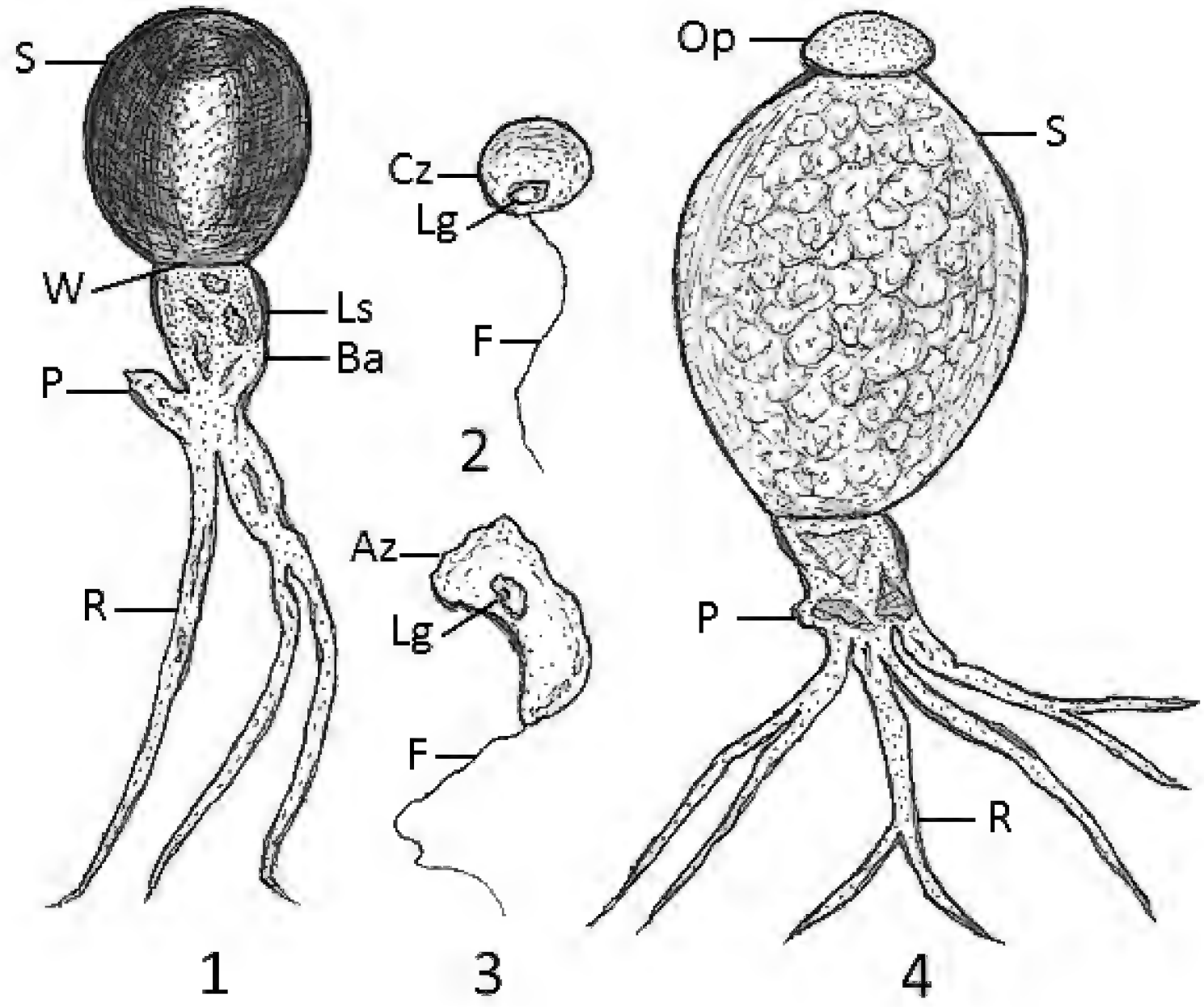

Figures 1-4. *Macrochytrium botrydioides*. **Fig. 1**: Young, darkish, sporogenous-portion of sporangium (S); transverse wall (W) delimits a lower, non-fertile portion of sporangium (Ls) which merges with the thallus-branch axis (Ba); prominence (P) at the base of the sporangial branch-axis represents the original, primary, vegetative thallus-apex which has been 'pushed aside' by growth of the sporangial branch; beneath this 'prominence,' coarse rhizoids (R) are evident. **Fig. 2**: Rounded 'chytrid'-form of zoospore (Cz); lipid globule (Lg) and single, posterior flagellum (F) evident. **Fig. 3**: 'Amoeboid'-form of zoospore (Az); lipid globule (Lg) and flagellum (F) still evident. **Fig. 4**: Mature, fertile sporangium (S), with distinct operculum (Op) beginning to be 'lifted' by pressure of zoospore-mass within sporangium; vestige of original thallus-apex (P) still evident on lower part of sporangial thallus-axis, rhizoids (R) below this. Figs. 1, 2, 4 after Minden, 1911; Fig. 3 after Minden, 1916. Additional useful illustrations, see Minden (1916), Tafel VIII, figs. 76-85; and Karling (1977), Plate 98, figs. 1-16.

Hybridization and introgression between *Juniperus communis* var. *saxatilis* and var. *hemisphaerica* in southeastern Spain

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ABSTRACT

An investigation of variation between *J. communis* var. *hemisphaerica* and var. *saxatilis* in Sierra de Baza, Cazorla, Mágina, Huéscar, S. Nevada (SE Spain) and Sicily revealed 5 SNPs and 1 indel (in nrDNA) that distinguished the varieties. Chloroplast (cp) petN-psbM did not clearly separate var. *hemisphaerica* and var. *saxatilis*, however trnL-trnF contained 4 SNPs and 2 indels that distinguished *hemisphaerica* and *saxatilis*. All plants from Sierra de Baza, Cazorla, Mágina, Huéscar and S. Nevada were var. *hemisphaerica* based on ITS data. Surprisingly, these var. *hemisphaerica* plants each contained var. *saxatilis* chloroplasts, suggesting chloroplast capture by an ancient evolutionary event. Hybrids (*hemisphaerica* x *saxatilis*) were found in four of the ten S. de Baza samples. Two plants from S. Nevada contained a SNP base characteristic of var. *saxatilis* indicating introgression. Published on-line www.phytologia.org *Phytologia 102(2): 83-87 (June 24, 2020).* ISSN 030319430.
KEY WORDS: *Juniperus communis* var. *saxatilis, J. c.* var. *hemisphaerica,* Sierra de Baza, Sierra Nevada, southeastern Spain, Baetic mountains, hybridization, introgression, nrDNA, cpDNA, trnL-trnF.

Juniperus communis var. *hemisphaerica* (J. & C. Presl.) Parl. was described from a shrub growing on the flanks of Mt. Etna, Sicily (Adams 2014). Analyses of nrDNA (ITS) revealed that its ITS differs from other *J. communis* varieties by 5 SNPs and 1 indel (Adams and Schwarzbach, 2013), making its DNA very distinctive among *J. communis* varieties. Yet, the discovery of additional var. *hemisphaerica* populations in Europe has been futile (Adams 2014). Prostrate juniper plants in Sierra Nevada, Spain, are often treated as var. *hemisphaerica*, but they are not small, globose shrubs as in Sicily. However, their ITS DNA is identical to that of var. *hemisphaerica* from Sicily (Adams and Espeut 2020).

Recently, DNA *J. communis* 'var. *hemisphaerica'* plants from the Pyrenees were analyzed (Adams and Espeut 2020). Nearly all the plants were prostrate and all but one had identical var. *saxatilis* nrDNA for the informative 5 SNPs and indel, except plant *15401*, that had 3 SNPs from *hemisphaerica* and 2 SNPs and the indel from var. *saxatilis* (Adams and Espeut 2020). Plant 15401 was judged to be a hybrid: *J. c.* var. *hemisphaerica* x *J. c.* var. *saxatilis*, despite the fact that no *J. c.* var. *hemisphaerica* were found from that population.

To investigate the more southern range of putative *J. c.* var. *hemisphaerica*, we initiated this study using nrDNA (ITS) and more chloroplast markers: petN-psbM trnL-trnF, trnS-trnG, and trnD-trnT.

MATERIAL AND METHODS

Specimens used in this study:
Juniperus communis var. *hemisphaerica*:
France:
Pyrenees: Lab Acc. *Robert P. Adams 15401,15402,15403,15404,* horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42.907° N 02.943° E, 600 m, Feb. 2018, coll. *Marc Espeut, ns 1,2,3,4*, 23
Pyrenees: Lab. Acc. *Robert P. Adams 15581-15600*(20),horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42° 54' 42.82" N, 2° 50' 37.47" W. 630 m, 8 March 2019, Coll. Marc Espeut ns 1-20, Lab Acc. all horizontal, except 15598 was a sub-shrub.
Spain:
Sierra Nevada: Coll. *Robert P. Adams 7194-7195.* prostrate to 0.5m tall, with *J. sabina.* Sierra Nevada, Granada province, Spain. 37° 06' 17" N 3° 24' 51" W, 2100m, 20 Oct. 1993.
Sierra Nevada: Coll. *Robert P. Adams 15702-15703* prostrate to 0.2m tall x 3-5 m wide, abundant at Ski area, Sierra Nevada, Granada province, Spain. 37° 06' 02.54" N, 03° 24' 00.55" W 2024 m, 6 June 2019,
Sierra de Baza: Coll. *Robert P. Adams 15692-15701,* with Carlos Salazar Mendias, Joaquin Altarejos, prostrate shrubs (0.2m high x 3 to 5 m wide) to very decumbent shrubs (0.5m x 3 to 5 m wide), common on limestone with *J. sabina.* Sierra de Baza, medium elev. site 2. 37° 22' 28" N, 02° 51' 03" W. 2024 m, 4 June 2019, Granada province, Spain
Sierras de Cazorla, Segura y Las Villas: Lab Acc. *Robert P. Adams 13052,* Coll. Joaquin Altarejos JA-089, shrub, 0.5m x 1 m wide. 37º 04' 34.16" N, 2º 55' 26.54" E, elev. 1,350m, 1 Oct 2011, Las Sierras de Cazorla, Segura y Las Villas, Jaén province, Spain and Lab Acc. *Robert P. Adams 13053,* Coll. Joaquin Altarejos shrub, 0.25m x 1 m wide. JA-090, 37º 55' 31.60" N, 2º 50' 25.13" E, elev. 1,350m, 1 Oct 2011, Sierra de Cazorla, Jaén province, Spain.
Sierra de Huéscar: Lab Acc. *Robert P. Adams 13054*, Coll. Joaquin Altarejos JA-091, prostate shrub, 0.1m x 1 m wide. 38º 01' 21.57" N, 2º 34' 48.05" E, elev. 1,350m, 1 Oct 2011, Sierra de Huéscar, Granada province, Spain.
Sierra Mágina: Lab Acc. *Robert P. Adams 13055*, Coll. Joaquin Altarejos JA-092, shrub, 0.25m x 1 m wide. 37º 44' 34.85" N, 3º 27' 10.82" E, elev. 1,350m, 1 Oct 2011, Sierra Mágina, Jaén province, Spain.
Voucher specimens are deposited in BAYLU and JAEN herbaria.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20º C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (cp markers) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS primers utilized. The primers for petN-psbM, trn: SG, LF and DT regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Chromatograms were processed by use of Chromas 2.31 (Technelysium Pty Ltd.).

RESULTS

Analyses of nrDNA (1270 bp) revealed an informative 3 bp indel at site 205 and 9 SNPs but only 5 SNPs were informative (SNP 3, site 400; SNP 4, site 404; SNP 5, site 414; SNP 8, site 642; SNP 9, site 1149). Samples from the Pyrenees (Adams and Espeut 2020) are included in Table 1, along with *J.*

Table 1. DNA variation in *J. communis* from southeastern Spain compared with other areas.

			ITS Indel[1]	ITS S3[1]	ITS S4[1]	ITS S5[1]	ITS S8[1]	ITS S9[1]	LF S1	LF S2	LF Ind	LF S3	LF S4	LF Ind	
coll. #	Location	ITS class	205	400	404	414	642	1149	160	208	473	503	521	622	cp class
11206	Norway	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
11207	Norway	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
7846	Sweden	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
7847	Sweden	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15402	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15404	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15590	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15593	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15583	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15586	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15587	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15595	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15598	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15592	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15589	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15581	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15582	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15584	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15585	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15588	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15403	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15591	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15594	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15596	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15597	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15599	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15600	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15401	Pyrenees	s x h	TTT	A	C	C	C/T	A/G	C	G	-	T	G	-	sax
15695	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15697	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15699	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15701	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15692	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15693	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15694	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15696	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15698	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15700	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15702	S. Nevada	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15703	S. Nevada	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
7194	S. Nevada	hemi	TTT	A	C	C	T	A	C	G	-	T	G	-	sax
7195	S. Nevada	hemi	TTT	A	C	C	T	A	C	G	-	T	G	-	sax
13052	S. Cazorla	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13053	S. Cazorla	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13054	S. Huéscar	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13055	S. Mágina	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
9045	Sicily	hemi	TTT	A	C	C	T	G	A	T	A	A	A	G	hemi
9046	Sicily	hemi	TTT	A	C	C	T	G	A	T	A	A	A	G	hemi

[1]ITS: ndel 205: xxxTGCTGGACGG; S400: GGACGTCCGx; S404: GGACGTCCGNGGCCx; S414: xTGAGATTT;S642: XGGGGCGGGG; S1149: xTCTTTGGTG. [2]cp trnLF S160: xAACATAA; S208: xAATTGTAC; Indel 473: ACACAATATx. [3]petN, S305: xGAACCATAC.

communis var. *communis* (Sweden), and *J. c.* var. *saxatilis* (Norway). These samples were uniform in their nrDNA that clearly separated them from *J. c.* var. *hemisphaerica* from S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, S. Nevada and Sicily. An exception from the Pyrenees was sample 15401, that had 3 SNPs from *hemisphaerica* and the indel and 2 SNPs from *saxatilis* (Table 1). The S. de Baza plants are divided into 2 groups: those which have typical ITS DNA, and four (15695, 15697, 15699, 15701) that are heterozygous at all six sites with nucleotides from both *hemisphaerica* and *saxatilis*, and TTT or - for the indel site 205(Table 1). These are hybrids. Two S. Nevada plants (7194,7195) have an A (ex *saxatilis*) at SNP 9 (site 1149), suggesting they may be introgressants with gene flow from *saxatilis* into their *hemisphaerica* nrDNA in the past.

Examination of the cp data (LF region) reveals every plant analyzed (Pyrenees, S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, and S. Nevada) has the *saxatilis* chloroplast! The only *hemisphaerica* cp is found in the plants from Sicily (Table 1). This appears to be a chloroplast capture evolutionary event as we have previously reported in *Juniperus* (Adams et al. 2017a,b, Adams et al. 2018, Adams et al. 2020, Farhat et al. 2019a,b, Hojjati et al. 2019).

A summary of plants in southeastern Spain and the Pyrenees shows (Table 2) that hybridization and introgression is common at S. de Baza (4 of 10 plants), and rare in the Pyrenees (1 of 29 plants). Yet, all plants analyzed have the *saxatilis* chloroplast, except the two *J. communis* var. *hemisphaerica* plants from the type locality in Sicily.

Table 2. Summary of hybridization and introgression in S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, S. Nevada and Sicily, plus two kinds of plants found in the Pyrenees. IG = introgressed.

coll. #	Location	nrDNA (ITS) classif.	cp class	Overall classification and status
28, all but one	Pyrenees	v. *saxatilis*	sax	*J. communis* var. *saxatilis*, no hybridization with *J. c.* var. *hemisphaerica*, except for 15401, below.
15401	Pyrenees	sax x hemi	sax	Hybrid x *hemisphaerica*, with *saxatilis* cp
15695	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15697	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15699	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15701	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15692	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15693	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15694	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15696	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15698	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15700	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13052	S. Cazorla	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13053	S. Cazorla	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13054	S. Huéscar	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13055	S. Mágina	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15702	S. Nevada	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15703	S. Nevada	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
7194	S. Nevada	*hemisphaerica*, IG *saxatilis*	sax	var. *hemisphaerica* introgressed by *saxatilis* (ITS), with *saxatilis* cp
7195	S. Nevada	*hemisphaerica*, IG *saxatilis*	sax	var. *hemisphaerica* introgressed by *saxatilis* (ITS), with *saxatilis* cp
9045	Sicily	*hemisphaerica*	hemi	var. *hemisphaerica* (ITS), with *hemisphaerica* cp
9046	Sicily	*hemisphaerica*	hemi	var. *hemisphaerica* (ITS), with *hemisphaerica* cp

It is unclear how *J. c.* var. *hemisphaerica* in Spain acquired the *saxatilis* chloroplast. Additional screening in Spain (and Europe) is needed to determine if all *J. c.* var. *hemisphaerica* have the *hemisphaerica* chloroplast, or if the phenomenon is local to southeastern Spain.

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Obituary and Tribute to Billie L. Turner: Botanist, Teacher, Mentor, Philosopher, Friend.

Robert P. Adams, Audrey Averett, Tina Ayers, Fred Barrie, Meredith Blackwell, Will H. Blackwell, Mark W. Bierner, Karen Clary, Piero G. Delprete, Wayne Elisens, Dorothy Irwin, Matt Lavin, David Northington, Richard Olmstead, Mike Powell, Susan Plettman Rankin, Peter H. Raven, Harold Robinson, Randy Scott, John L. Strother, Tod F. Stuessy, Spencer Tomb, B. L. Turner II, Matt Warnock Turner, James Walker and George Yatskievych

This is a compilation that includes the obituary of Dr. Turner, printed in the Austin Statesman, 14 June 2020, plus memories by students, colleagues and friends of Billie, slightly edited (but not censured) by Robert P. Adams, ed., Phytologia, Biology Department, Baylor University, Waco, TX, 76798, USA, robert_adams@baylor.edu, Published on-line www.phytologia.org *Published on-line www.phytologia.org Phytologia 102(2): 88-105 (June 24, 2020). ISSN 030319430.*

Billie Lee Turner

Billie Lee Turner, age 95, Professor Emeritus of the Department of Integrative Biology at The University of Texas at Austin and a long-time resident of the capital city, passed away May 27, 2020 in Round Rock after several years of declining health and a bout of COVID-19. He held the Sidney F. and Doris Blake Centennial Professorship in Systematic Botany until his retirement in 2000.

Billie was one of the nation's foremost plant taxonomists, having propelled biochemical systematics—using chemistry to classify plants—to the forefront of the field where, before the advent of DNA, it remained the vanguard of plant classification for over 20 years. He was particularly known for his expertise in the *Asteraceae*, or sunflower family, especially in the U.S. desert Southwest and Mexico.

Billie served as secretary to the Botanical Society of America (1959-64) and later as vice president (1970). He was president of the Southwestern Association of Naturalists in 1967 and had membership in 10 or more U.S. and international societies. At UT he chaired the Department of Botany (1967-74) as well as the Division of Biological Sciences (1972-73), a period in which the program solidified its status as a prominent center for botanical research. He won the leading research and teaching accolade of the American Society of Plant Taxonomists, the Asa Gray Award, in 1991.

With a publishing career spanning seven decades, he authored over 700 scientific reports and articles, naming over 1.400 plant species, varieties, and new combinations. During a half century of teaching, he mentored or served as the major professor to approximately 25 masters and 60 doctoral students, many of whom became distinguished academics. He quintupled the holdings of the UT herbarium, turning it into a world class research facility.

Born in Yoakum, Texas, on Feb. 22, 1925 to James Madison Turner Jr., and Julia Irene Harper, Billie spent his earliest years in Sanderson and always considered the Trans-Pecos his ancestral home. Following his father's railroad job, the family lived for a short while in Dunlay before settling in Galveston around 1930. Surviving childhood in that island city during the height of the Great Depression was, according to his many stories, a chaotic and exhilarating affair of debt-fleeing moves, dog bites, divorce, and deprivation, set against a ribald backdrop of speakeasies, honky-tonks, gangster-run casinos, and crime.

The family's move to Texas City in 1939 brought much-needed stability. Billie participated in football and track for the Central High Stingarees, while doing janitor work in the evenings and reading the works of Shakespeare. He graduated valedictorian in May of 1943.

A month later, he enlisted in the Army and transferred to the newly created Army Air Corp. By the time he finished navigation school he was among a handful promoted to officer rank (second lieutenant), and by Christmas of 1945 he had joined the 15th Air Force division stationed at the Giulia Airfield in Cerignola, on the east coast of Italy. He served as navigator on B-24s, making bombing runs on Austria and Germany, during which he was awarded the Purple Heart when his was the only plane in the squadron to return from a hell-raising sortie over Brenner Pass. An emergency landing in Switzerland on his 17th mission pulled him out of active combat. He was later stationed in Heidelberg and Straubing, Germany during occupation where he was promoted to first lieutenant.

In spring of 1947, before his military service had officially ended, Billie was so eager to start college that every Sunday he snuck away from El Paso, where he was stationed, to Sul Ross State University in Alpine, to begin his studies, returning to El Paso every weekend for muster. He was aiming to be a lawyer until taking a certain class with Barton Warnock, beloved authority on West Texas flora at the time, forever changed his career trajectory to botany. Taking full advantage of the G.I. Bill, he amassed three degrees in six years: BS Biology, Sul Ross State University (1949); MS Biology, Southern Methodist University (1950); and Ph.D. Botany, Washington State University (1953).

Billie began his academic career as an instructor at The University of Texas at Austin in 1953. An auspicious trip to Africa in 1956-57 with Homer Leroy Shantz, former president of the University of Arizona and arid lands expert, moved his post to tenure track. With the publication of their *Vegetational Changes in Africa over a Third of a Century* (1958), along with his first sole-authored book, *The Legumes of Texas,* a year later, Billie rose to associate professor in fall of 1959, and two years later was promoted to full professor. His skill in using chromosome numbers, and especially chemistry as a tool to classify plants, culminated in the benchmark *Biochemical Systematics* (1963), co-authored with his colleague Ralph Alston. Other noted works include his *Plant Chemosystematics,* with J. B. Harborne (1984), *Atlas of the Vascular Plants of Texas* (1987), and *The Comps of Mexico: A Systematic Account of the Family Asteraceae* (27 volumes, 1996-2017).

An avid field collector, Billie instilled in his students the importance of knowing how species behave in nature. His personal collections, numbering well over 10,000 specimens, informed his research, and much of his heart was centered on UT's herbarium, which he helped grow from 200,000 specimens in 1967 when he became its director, to one million specimens by the time he stepped down in 1998. Since 1984, the collection and facilities, which the university recently named the Billie L. Turner Plant Resources Center in his honor, has been housed in UT's iconic tower, a location that he took pride in having negotiated. The collection ranks fifth among U.S. university herbaria and twelfth across the nation. Its holdings from Texas, Mexico, and northern Central America are world class.

By any reckoning, Billie was a character. Naturally cheerful, optimistic, and gregarious, he was welcoming to anyone who showed the slightest curiosity in the world, and even to those who did not. He was as interested in people and their quirks as he was in plants, and he was magnanimous to his students with his time, support, and pocketbook to ensure their success in what he thought was the best profession in the world. But he also did everything his way, mocked the status quo and social mores, was honest to a fault, and lacked the filters that many see as needed for civil discourse. His flamboyant innuendos and rakish behavior got him called into his dean's office on several occasions. It was a point of honor that he survived the (alleged) attempts by three different university presidents to fire him.

Billie is survived by two sons from his first wife Virginia Ruth Mathis: Dr. Billie L. Turner II, of Fountain Hills, Ariz., Regents Professor and Gilbert F. White Professor of Environment and Society, Arizona State University, member of the National Academy of Science, and his wife Carol Snider; and Matt Warnock Turner, Ph.D., of Austin, writer, market researcher, and instructor in UT's Liberal Arts Honors Program. He is also survived by adopted sons Robert Lee Turner of Austin and Roy Parker Turner of Dublin, Calif., children of his third wife Gayle Langford, of Santa Fe, New Mexico. Billie is further survived by his granddaughter, Victoria Kelly Turner, Ph.D., assistant professor at University of California Los Angeles; great-granddaughter, Siena Leigh Turner-Rudy; many nieces and nephews in Texas, Alabama, and West Virginia; and by his beloved and devoted personal friend of many years, Jana Kos of Austin.

A celebration of his life will be arranged at a later date. Donations in Billie's memory can be made to the herbarium that was his life's work and to which he bequeathed a large part of his estate: Billie L. Turner Plant Resources Center, c/o University Development Office, The University of Texas at Austin, P.O. Box 7458, Austin, TX 78713-7458, or simply use the link: txsci.net/billieturner

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On May 27, 2020, 2 pm (Austin), Billie Lee Turner passed away at 95 years young. The immediate impact was traumatic to his former students and friends. In an effort for us to pay tribute to his life, I asked his students and friends to send me a special memory they have of Billie (always will be Dr. Turner to me). Below are brief paragraphs from colleagues, friends and students that I hope will give the reader (including me) a more complete picture of this unique 'bigger than life Texan'.

The following two memories are by Billie's two sons, Matt and Billie II and give their recollections of their dad.

B. L. Turner II, Ph. D.

Big Billie was the family name until Dad divorced Mom. I was Little Billie. Throughout my youth—which included Dad's entire higher education and his early professional career at U.T.—Big Billie was not the personality he would later become, at least not completely. He was the consummate father, a cross between a playground friend-coach and an academic-intelligentsia version of "father-knows-best."

Dad always rose late, after I departed for school, but came home at 5 pm for dinner, after which he would attend to my desire to be an athlete, before he would depart back to the herbarium. With Ramsey Park five minutes away, he would hit fly balls to me, which gave me a leg up to make Little League early. On weekends, the neighborhood kids would come over to attend to Mr. Turner's track-and-field "school", where we were taught the "western roll" for high jumping and engaged in races on Ramsey Street, which invariably culminated in an around-the-block, long distance event. Big Billie enjoyed such engagements even more during my teen-years as sport moved to football. In late August, before junior-high classes began, the gang would migrate every evening to the lighted park for tackle football. Dad often played. He had a shoulder injury which prevent him from throwing well overhand, so he invented an underhanded, sidearm technique, akin to a discus throw. He was pretty accurate and could heft the ball about 30 yards! After the game, he often loaded all the boys in our car for a trip to A&W and a root beer float. Then back to the herbarium he would go. He came to every sporting event in which I participated but he never really encouraged me to be an athlete—perhaps he observed what I did not want to admit, my level of athletic abilities. His real legacy as a Father, however, was not the playground—although he was the only Father ever there—it was the extensive time he spent as an intellectual and moral mentor. Big Billie did not believe in children books. He read to me most nights, focused first on poetry, advancing from "Little Boy Blue" to "If" and "Ozymandias," before elevating to Shakespeare, foremost Hamlet. By 5ish I could recite "to be or not to be." Such readings were not be read alone; each line elicited a question for me to answer. This challenge to think never ceased. A typical dinner conversation began with a pronouncement. The two I most vividly recall were: "A recent study identifies

that the little toe is the sexist part of the human body"; and, "A nuclear missile was launched this evening from Cuba to the East Coast." It was then up to Little Billie to argue why such claims were nonsense. Beyond such proceedings, were constant articulations of the significant qualities that individuals should develop and maintain—to be the person identified in Kipling's poem. I can't possibly count the number of times I was told: "I don't care if you are a ditch-digger as long as you are the best ditch-digger you can be, and are honest and happy." His decision to divorce Mom generated the most difficult discussions between us. In retrospect, I understand these were justifications of his decision and an effort to gain some small measure from me that the decision was correct, or at least understandable. At the time, however, these discussions were embarrassing to me, and I failed to respond as Big Billie would have wished, with empathy and insight. Dad changed in many ways during my college years, especially in the time and attention given to family, and in taking on the characteristics that Matt identifies. Yet another entire story is required for that part of his life.

Matt Warnock Turner, Ph. D.

Dad was compulsively curious about people. "Too soon made glad," he'd call it, with a nod to the poet Robert Browning. He wanted to know their stories, what idiosyncrasies they had, or what passions ruled them. Colleagues were daily fodder, but strangers were a special treat, and women a delicacy. The first great taxonomic question—as if he were separating monocots from dicots—was always, "Are you married?" And with their answer, the great dichotomous key would begin. If married, what do you think of your spouse? (Love them deeply? Marriage of convenience? Ready to trade them in?) If not married, why not? (Haven't found the right person yet? Divorced? Gay? Monastic?) Each answer led to new binaries, and by the end, I think he had sorted them into some sort of species, or at least variety. This *performance*—in gentle mock I called it "The Billie Turner Show" and would start to hum the theme music to the Tonight Show with Johnny Carson—rarely caused offense, such was the power of his unaffected humor and white hair. The act usually ended with a standing invitation to stop by the Herbarium, Main 127, in the Tower, you know, THE TOWER. If they actually did show up, they'd get a brisk welcome, a mini-tour, and an invitation to date whatever poor soul was mounting specimens that day. He sometimes later complained, "I thought I'd never get rid of them."

In my more cynical moments, I would seize upon these words as proof that Dad's obsessive inquiries were mere egotism. He really wanted to talk about *his* life, *his* marriages, *his* hard-scrabble upbringing. YOU merely provided a venue. Yet, as I peruse the letters and cards that flood in from his former students, colleagues, and friends over the past weeks, I see that I am largely mistaken. Your tributes attest to his sincere devotion: "Your dad launched my career;" "He found me this job;" "If it weren't for him, I wouldn't have met my spouse;" "After my parents, he is the most important person in my life;" "He changed my life forever." Clearly YOU were loved after all, each of you, individually. His penchant for naming species after people, rather than traits or geography, reflected this. And even you strangers…he might have forgotten your names, but without fail he remembered your faces, years later, as if you had been corollas collected once, long ago, in a sun-drenched ravine in Durango. If Dad's curiosity was compulsive, his inquiries obsessive, his heart was always in the right place. His attention to uniqueness, whether in plants or humans, left the world a brighter place.

The following memories are by Billie's graduate students, colleagues and friends, listed in order of the date when they first came to know Dr. Turner.

Dorothy Irwin, younger daughter of Howard Irwin (deceased), Ph. D. 1960, B. L. Turner, UT Austin.

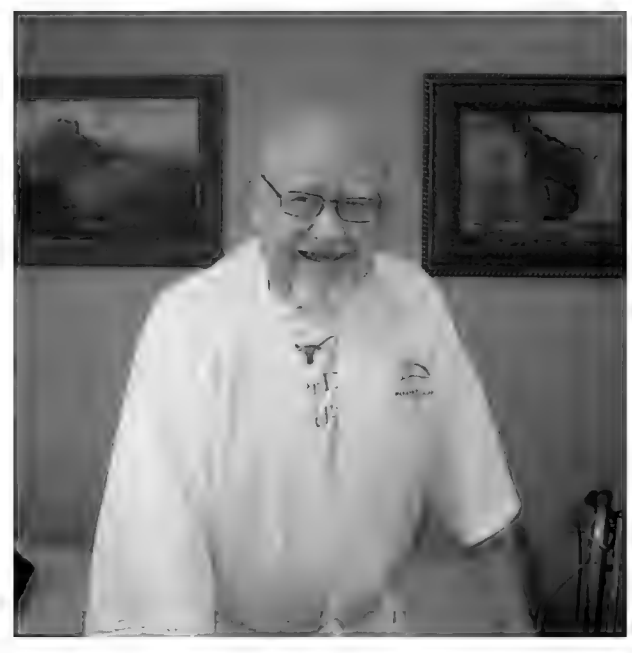
In 1954, Howard Irwin began his second year as a Fulbright instructor teaching biology at a boys' school in British Guiana. He had just started to search for a graduate program at a U.S. university through which he could continue pursuing his newfound interest in tropical botany, specifically *Cassia*. His prospects brightened in April when he began a correspondence with Dr. Billie Lee Turner at the University of Texas in Austin. It was a propitious connection since, as Irwin's wife, Marian, wrote to her

parents in the States, "some of the botanical groups that are found here [in the South American colony, now Guyana] are also found there—different species, but the same families." Turner "suggested an intensive study of one family both here and in Texas, then forming a thesis around the conclusions." The two men, who were just a few years apart in age, met in June. To make the trip, Irwin flew from British Guiana to Trinidad, then to Panama, on to Houston, followed by Dallas, and finally to Austin. Turner met him at the airport. By the following year, Turner was mailing microfilm about *Chamaecristae* to Irwin for him to examine. In return, Irwin sent a series of vials containing plant buds in a fixative solution. "Dr. Turner writes nice letters, mostly business," Marian wrote her parents in March 1955. "In this one he said he was sure we'd like Austin, that it's uncivilized enough for most people to be genuinely friendly, yet civilized enough to have most of the amenities." And the following year, to Austin the Irwins went.

Turner and Irwin collaborated on two journal articles published in 1960, in *American Journal of Botany* and *Rhodora*. Upon his commencement from UT that spring, Irwin was hired by the New York Botanical Garden, where he worked for the next 18 years, rising to its presidency in 1973. He and Turner stayed in touch, and when Turner and his wife visited New York City in the 1980s, they paid a visit to Irwin and his second wife, Anne, in Huntington, on Long Island. Irwin's Ph.D. had been bestowed with a gift: an original 1816 edition of Colladon's *Histoire Naturelle et Médicale des Casses*, which, following Irwin's death last year, is now in my collection. The book is inscribed: "To Howard, for a job well done! B. L. Turner, 1960."

James ("Jimmy") Walker, Ph. D. 1969, Harvard, Botany Undergraduate at UT 1961-64, B.A. in Botany with High Honors 1964,

I first met Billie the summer, 1961, before I was to enter UT as a freshman botany major. I knocked on his office door and said that I was interested in angiosperm systematics. After telling him all about my desire to become a plant systematist he said: "My God young man you know more already about what you would like to do than most of my graduate students! Come with me and I will give you a desk in the herbarium that you can use while you are a botany major". From that moment on Billie took me "under his wing" and treated me like one of his graduate students. I particularly enjoyed working with him to identify new herbarium specimens to family and sometimes even to genus. I went on a field trip to Mexico with him and some of his graduate students (see Billie's book "All My Academic Children," p.73). I often went with him and Ralph Alston to have coffee in the cafeteria near the Botany Building. It was Billie who suggested I get into the Plan II Honors Program, which I did. Having a one-on-one interview with John Silber, who was the Director of the Plan II Honors Program, is a story in itself. Billie also wrote a supporting letter that helped me get elected a Junior Fellow in the Society of Fellows. John Silber was the Head of the Society of Fellows which is also another interesting story, particularly since that story involved both Billie and John Silber. When I got inducted into Phi Beta Kappa, I invited Billie and his wife Ruth to the induction ceremony and dinner instead of my own parents. If he had not been in my life I would never have gone to Harvard for my doctorate and had my career in systematic botany, although that story involves Harold Bold as well. I have so many memories of Billie. I loved it that I was able to have him as a visiting professor for a semester when I was a faculty member in the Botany Department at UMass/Amherst. Over the years Billie and I always kept in touch by phone, and he must have sent me dozens of clippings from my hometown newspaper the "Taylor Daily Press". I am so glad that I was able to have a nearly two-hour long talk with him in his nursing home in Austin when I visited Texas for the first time in more than 20 years in June, 2017. Talking with Billie then was the highlight of my trip back to Texas.



Billie Turner in his extended care home in Austin, Texas, June 26, 2017

Billie Turner was undoubtedly one of the most important people in my life.

Peter H. Raven, President Emeritus, Missouri Botanical Garden (event - summer, 1963)

From the time I visited Billie Turner in the summer of 1963, searching for evening primroses in Texas, I loved him. He welcomed Dave Gregory and me to his home, and we started a warm relationship that lasted from then on. His own difficult youth experiences probably helped to make him supportive of those around him, encouraging them in any way that he possibly could. He unfailingly greeted others with a broad smile and encouraging words and plans. Obviously disliking posturing of any kind, he used gruff words to shake up people if he considered them too serious or self-centered, and that habit made some people dislike him, misunderstand him, or be afraid of him. He was very proud of the fact that his two sons earned Ph.D.'s and excited at the later success of his granddaughter Kelly, who earned a Ph.D. at UCLA.

Billie once told me his story about Texas really being five countries, the other four defined by specific inborn dislikes, but ending with "Right here in Central Texas we are fortunate to have the most loving place on Earth." Billie certainly was a major contributor to keeping Austin that way, as generations of students, faculty, and neighbors will affirm. Joining the faculty of the University of Texas – Austin when he was 28 years old (1953), he worked for nearly 70 years to help make it the fine academic center that it is today. The many people he encouraged over the years will rightly honor his memory and remember his smile, as I certainly shall.

Mike Powell, Ph.D. in Botany, 1963, B. L. Turner, UT Austin.

I am forever grateful that in 1960 Professor Turner accepted me as one of his graduate students in systematic botany, and that he remained a mentor and friend throughout his life. Off and on for ca. 40 years, Professor Turner and I collaborated in a number of research projects, some of them long-term, including a series of papers dealing with chromosome numbers in Compositae, and the study of plant species endemic to gypsum exposures in northern Mexico and parts of the southwestern U.S. Some of my favorite memories of Billie Turner are associated with our numerous field trips, usually with other botanists and friends along. In the early 1970s we made two trips to Baja California, Mexico, collecting and camping, where particularly memorable activities included camping in a boojum (*Fouquieria columnaris*) forest near Bahia de los Angeles, and farther south stopping for a day and night at Bahia Conception (Fig. 1), where Billie's somewhat embellished improvisation, the "Baja Olympics" took place (see pp. 16-17, Turner, B.L. 2015. All My Academic Children, Texensis Publishing, www.phytologia.org). Most trips to Mexico took us east of Sonora, Sinaloa, and Durango (Fig. 2), at many gypsum sites in northeastern states, including the magnificent white gypsum dunes near Cuatro Cienegas, Coahuila, Billie loved to get in the field, and to me he was exceptionally perceptive in recognizing (or imagining) populational variation. "Probably this is a new species" he would proclaim excitedly, sometimes more than once in a day. Field excursions with Billie were always inspirational botanical experiences

Fig. P1. Prof. B. L. Turner, 1972, in camp at Bahia Concepion, Baja California Sur, Mexico.

Fig. P2. Prof. B. L. Turner, year not certain, possibly early 1970s, Durango Mexico, posing with *Perityle turneri* (Asteraceae), one of many species named in his honor.

Will H. Blackwell, Ph. D. 1967, M. C. Johnston, UT Austin.

I was a student of Dr. Marshall C. Johnston during my doctoral training at the University of Texas (Austin), 1963-1967. I will always be appreciative of Marshall's superb mentorship throughout my dissertation work on *Bouvardia* (Rubiaceae). But the present little essay concerns interactions with Dr. Billie L. Turner during that time period. I remember coming back to work, many nights, during my stay at Texas, particularly (of course) to work on my seemingly unending dissertation project; the 'toil' and 'tedium' in such a project could surely weigh heavily on a person, as it did on me at times—the 'finish-line' always seeming 'out there,' indefinitely in the future somewhere (I actually graduated on schedule, 1967). Anyway, my spirits, during the potential drudgery of such night-work, were frequently bolstered by the bright spirit of a professor also coming back to work nights in a nearby location associated with the herbarium—This person was Billie Turner, who was working, most effectively, on 'one Composite genus after another' (with 'no end in sight,' and no wish for any). Just seeing a professor still so excited to personally do research gave me not only a lift in spirit, but confidence that this sort of work could (would!) have good outcomes—perhaps for a lifetime. Dr. Turner would often stop by my work-station saying something like, "Keep up the hard work, kid; you're going to get there!" This seemingly small thing meant so much to me in maintaining the perseverance it took to complete my dissertation, and to go on to be a 'practicing taxonomist'—even though my efforts (still taxonomic) eventually shifted back to algae and fungi (reflecting initial interests). I will always be grateful to Dr. Turner for giving me, time and again, those little reinforcements of inspiration that I feel proved vital to my success at Texas, and later as a professor, Miami University (Ohio). After retiring (emeritus) from Miami, I went on to further 'practice taxonomy' as adjunct professor, The University of Alabama. Thoughts of Dr. Turner's great enthusiasm for botanical research are still an inspiration. In each taxonomic project I work on—no matter how difficult—I can hear him say, "You're going to get there!"

John L. Strother, Ph. D. 1967, B. L. Turner, UT Austin.

The Dr. Billie Lee Turner I knew and studied with (1964--1967 and beyond) was smart and enjoyed proving it. He especially enjoyed "testing" newly-met people. In getting to know a newly met person, Dr. Turner would ask questions related to topics that might have two or more sides (politics, religion, sports, et al.), if he got a sense of which "side" a newly met might favor, he would proceed to defend an alternate position, seemingly trying to persuade the newly met that they were on the "wrong" side. I suppose that was Dr. Turner's way of quickly assaying a newly-met's intellect.

And, if someone questioned a Turner taxonomic position, he would strongly defend his position: pointing out differences that supported his splitting a taxon into two or pointing out similarities that justified his lumping two taxa into one, voice and vigor rising higher and higher, sweat breaking out on his forehead.

An instance: species circumscriptions in *Schistocarpha*. He being the "splitter" and "I being the lumper." Would we should all be so passionate about our work.

RIP Dr. Turner.

Tod F. Stuessy, Ph. D. 1968, B. L. Turner, UT Austin.

I arrived in the Department of Botany at Austin in June of 1965, a neophyte with a B.A. from DePauw University, a small liberal arts school in Greencastle, Indiana. I had worked for two summers in the herbarium of the Field Museum, which had given me some idea of what research in systematic botany was all about, but only at a very general level. During my first week in the herbarium in Austin, Dr. Turner (he was always Dr. Turner to me until many years after graduation) took me into the hallway, opened up a cabinet and said: "Here is the genus *Melampodium* (Compositae), and I think it would be a good thesis project for you." I, knowing nothing about this, said simply: "OK, sounds good to me." And that was that. He had neglected to mention that a previous student, Robert Merrill King, had started on the group and left the program, but the problems were due primarily with personality issues, as I learned later. The genus with about 36 species was very suited to my abilities and interests, among which included traveling in Latin America. Dr. Turner was an excellent major professor in several significant ways. He

was not an organized classroom lecturer, nor was he always available for serious consultation about research or career issues. This was in part because during my years in the program, he was undergoing his divorce from his first wife, Ruth. He was now in a new world of relationships, and he was fascinated by what he was learning. This was understandable, but it was easier to get a conversation with him about love problems than about the systematics of *Melampodium*. I did not enjoy the "coffees" in the Student Union, which often revolved around embarrassing personal questions about other graduate students. For career guidance, Tom Mabry and Marshall Johnston both were willing to counsel students, especially Mabry, and they filled in this important need. But Turner achieved a very important thing--he instilled confidence in his students, supporting us and letting us believe in ourselves, which is one of the essentials for success after graduation. We learned from him by watching his discussions in meetings and seminars, seeing him shuffling sheets in the herbarium late at night, and sensing the excitement and challenge that systematic botany offered.

One specific event may be worth relating. In Spring of 1968, I was scheduled to defend my Ph.D. thesis in May, followed by getting married in Austin in June. My parents came down from Illinois for the wedding, and in a spirit of appreciation, asked me to invite Dr. Turner to dinner. When I had graduated from DePauw, they had done this with my undergraduate mentor, Dr. Preston Adams (a student of Reed Rollins from Harvard), and it worked wonderfully. Well, I dutifully invited Dr. Turner to dinner and he accepted, much to my dismay. This to me was going to be "The dinner from Hell." Turner at this time loved to ask very embarrassing sexual questions just to see how persons would react. My father was not pugnacious, but he was proper and conservative, and I could only imagine how badly this event would turn out. On the appointed evening, Turner arrived at the restaurant well dressed, and he miraculously was polite and gracious throughout, resulting in a very lovely evening, giving pleasant memories for my parents. They related afterward how engaging he was in conversation, and I was relieved that this was the only side of Dr. Turner on display that evening.

Summing it all up, I am extremely appreciative of having had the chance to study and work under the guidance of B. L. Turner in Austin. He was a dynamic role model, the research environment within the department was excellent, and the other students were highly motivated and helped me a great deal, especially John Strother and David Flyr. I asked so many questions of these two in the first year that John finally said: "Stuessy, are you also going to ask me to write your thesis for you?" It was a great start on a career in systematic botany, and I feel lucky to have been given the opportunity to apprentice under this brilliant but very untypical professor B. L. Turner.

Meredith Blackwell, PhD, 1968, CJ Alexopoulos (1964-1968, *1970-1973); BLT plant speciation class, 1967.*

Billie Lee Turner, outrageous friend, May 28, 2020

There's a lot to remember:

hour quiz 2 (Bot 392) with question on a *Baptisia* PNAS paper, discuss "Alston and Turner claimed …", saved since 1967 for the note, "fine paper, thank you"; coffee some Saturday mornings when no one else was there to go; the day in 1971 when he learned David had died; occasional parties, one when Stafleu drank some of my beer in the kitchen; distinguishing junipers by fungus-like splotches on bark; a beaded curtain; the early morning he helped Mrs. Alex out of a dumpster where she'd searched for letters, thought thrown out in her husband's office clear out; encountering his father and brother, popping out from behind a 2nd floor hallway incubator; his vault over a 4-foot post, saying, pretty good on my 50th birthday.

Because of a photo he showed of *Baptisia* shielding a cottonmouth, I planted some in my yard three years ago. I thought of him yesterday as I looked at those plants, noticed one lagging far behind the others, despite the rain; today I heard, yesterday he'd died. Billie wrote a memorial poem for someone close, "Friend, first day dead" (February 18, 1967). The poem, kept in my various office desks more than fifty years, now is locked in a building, closed by a virus. Fortunately, Johns Hopkins University Press granted permission[1] for Phytologia to reprint the poem:

Friend, First Day Dead*

Friend, first day dead,
This is the place
We used to have coffee;

You sitting there, just opposite,
Finger to temple immersed
In your own thought-logic
Discussing what counts
In life and what doesn't count,
High-planed rhetoric yours;
Mine the smile and flippant phrase
Designed to ease so much seriousness,

I miss you friend
This morning over coffee,
Your silence now the deepest
Philosophy yet, and
How can I answer the
Question you pose?

Friend, first day dead,
You take unfair advantage'
Were it me instead.

*Ralph E. Alston, 41, professor of botany at the University of Texas, died suddenly February 17, 1967.

[1]**Guthrie, R.D. and B.L. Turner. "The Ethical Relationship Between Humans and Other Organisms." Perspectives in Biology and Medicine 11:1 (1967), 62. © 1967 Johns Hopkins University Press.** Reprinted with permission of Johns Hopkins University Press.

Robert P. Adams, Ph. D. 1969, B. L. Turner, UT Austin (1966-1969).

Although, I thought that I was pretty unusual as a student entering the Ph. D. program (with about 70 graduate students in the Dept. of Botany, ranked as number one in the US), in hindsight, most of us were unusual. My introduction to 'Prof. Turner' was his class in General Biology when I (as a senior math major) was forced to take Biology in order to get a BA in math at U. Texas, Austin. Prof. Turner really made genetics and plant adaptions so interesting that I thought, "If I had it to do over, I would have majored in biology". But, I was ready to graduate in math/ computer science and it was not until four years later: 2 years computer programming in California and 2 years farming in the Texas Panhandle, that I was back at UT, enrolled in grad school in Botany. Billie was on sabbatical on safari with Homer Shantz in Africa that spring. When he returned in the fall, I went to his office to meet him. He (of course) said "Sure, I remember you". Even if not truthful, if still made me feel good. The Botany department had accepted me with only 2 semesters in freshman biology. So, I knew nothing. Billie was interested in me because in 1966, very few botanists were math - computer science majors! One day when we went to 'coffee' at the nearby student union, and he asked me if I could write a computer program to analyze data over a large geographic area (as I later learned, Billie was very interested in clinal variation within a species). I said "yes", and we immediately went up to the herbarium on the 4th floor. He pulled out a specimen of *Cercis canadensis*. Billie said 'I have noticed there is variation in the morphology of redbuds from Austin, up through Oklahoma and beyond'. Billie paused, kind of smiled that mischievous way, then said, in true Texanese style, "No, that won't work. Those damn Oklahoma

highway people have planted it all along the highway. You won't know what is natural or planted". Then Billie said "You need to study a common tree that is easy to find, and you could sample at regular intervals." And he said, "There is an interesting problem in *Juniperus*. Dr Hall says *Juniperus ashei* and *Juniperus virginiana* hybridize, but I don't think they do. Do you know what a juniper is?" I guess I had a 'deer in the headlights look', so he asked "Do you know what a cedar tree is?" Of course, being from East Texas, I knew that. We went back for 'coffee' the next day, and he took a table napkin and drew out the distributions of *J. ashei* and *J. virginiana*, put some Xs, equally spaced, across the range of *J. ashei.* And that was the end of Thesis 101. Thesis 102 was a practical lesson I have never forgotten. A few days later, Billie told me "You can do lots of computer work using morphology, but that won't get you ahead of other good candidates in getting a good position. You need to do chemistry also. Dr. von Rudloff has just published a paper on volatile leaf oils of juniper and he has quantitative data for each terpene." So, that started my long love affair with both *Juniperus* and terpenes/ mass spectrometry and, recently, DNA sequencing. End of Thesis 102 lesson, and a special insight into Billie's care for his students to try to prepare them to succeed. In some ways, Billie was an enigma, just as the blind men describing an elephant by feeling the tail, trunk, ear, etc. Aside from Billie usually opening most meetings asking about my sex life (which I soon learned to say 'fine', else the conversation would be way too long), I found him to a good mentor as he 'took me under his wing' as a very naive graduate student, but gave me tremendous freedom to pursue my thesis and continued to encourage me throughout my academic career. Some of the most precious memories have been working with Billie to publish his 'Comps of Mexico' series from 2007 - 2017 (ending just 3 years ago), when he began to complain 'My memory is getting so bad', but, together, we succeeded in publishing 19 volumes in the series. Especially, later, from about 2015 through 2017, Billie would always finish a telephone conversation by saying 'I will be eternally grateful for you helping me.' And, I can say the same about him. Billie often said "I hope my last academic words might be, while examining a batch of comp specimens: "be damned if its not new to Science!"'

Harold Robinson, Ph. D., Smithsonian Institution

I was not a student of Dr. Turner, but I was drawn into the study of Asteraceae by a person who was his student, R. M. King. Our interaction with Billie was an odd one since we saw so many flaws in the classification of the Compositae at that time, while Billie and Arthur Cronquist were both rooted in the Bentham system. We had to publish in the, then, unreviewed Phytologia since Billie and Arthur and their followers would have blocked publication of our papers in any reviewed journal. Nevertheless, in later years, Billie and I exchanged friendly messages, and I accept that Billie had a brilliant mind that he later began to use constructively. In fact, while he helped educate me through Bob King, I felt I later returned the favor by providing him with the basis of his later work. It is interesting to consider what he might have done with DNA sequencing. You know, I might even name a Comp after him some day.

Spencer Tomb, Ph.D. 1970, B.L. Turner, UT Austin, 1967-1970

My three years in Austin as one of Billie Turner's graduate students was a special time in my life. I was in a great bunch of grad students. Professor Turner loved life, his work and his students. From the first day I met him and throughout my career, I felt he was genuinely interested in my success.

We often went to coffee in the Student Union with Dr. Turner. He loved to jump on the banister side saddle and slide down and jump off at the right time and then hop on the next banister and leave us behind. One time he jumped off on the second floor just as Dr. Arnott appeared, reading something. Billie brushed the papers out of his hands and jumped on the next banister as he said, "Hello Howard." He left us to help Dr. Arnott gather his papers. It was hard for us to keep a straight face.

In 1969, Dr. Turner went through the entire herbarium looking at every specimen. Over the years, some specimens had been misfiled and others had been accessioned to the herbarium that were useless. The job was like one of the labors of Hercules. You could tell where he was working by the full trashcans. We pasted a Playboy centerfold on a herbarium sheet and put it ahead of him. Two days later the sheet with the Playboy Playmate appeared in the herbarium prep room with a thank you note. It said, "Thank

you for reminding me what is important in life." We put several more ahead of him after that and he left a note when he found them.

While Dr. Turner was on a collecting trip a new refrigerator was delivered to the herbarium. We leveled it and transferred what was in the old refrigerator, but we kept the crate, and put it in Dr. Turner's office, making it difficult for him to get to his desk. We got one of the secretaries to write a note saying, "Dr. Turner: We were not sure where this refrigerator was supposed to go." The next morning he returned you could hear him complaining. "Dang, I am gone a few days and I come back to this. It will take three men and a dolly to get this to the herbarium. It is going to cost the department money." I made my way through the small crowd that had gathered to see what he was whining about. I told Dr. Turner that I can get it up to the herbarium. "You can't move this up there," he said. I picked up the crate and walked out of the room. He flopped into his chair grinning from ear to ear.

Near the end of my dissertation defense, Dr. Turner asked me if I thought I was trained and prepared for the 1970s. I said," Yes sir; I think I am." He did not like my answer because new methods and new ideas were appearing all of the time. I explained that I had an open mind and I could adapt to the changes. The committee supported me and the next time I looked at Dr. Turner he had tears rolling down his cheeks. I thought I had hurt his feelings. I apologized to him that evening. He explained that he was pleased at my defense, but very sad because I was the first of his students that had defended without a job or a post doc. He felt he had let me down.

Audrey Averett (widow of John Averett, Ph. D. 1970, B. L. Turner, UT Austin, John passed away January 1, 2017).

One incident I recall really showed Billie's tenacity and competitiveness on the tennis court. As soon as he learned John had some tennis championships and a cupboard full of trophies to his credit, Billie (some twenty years older) saw a challenge. He and John spent many lunch hours and frequent weekends on the tennis court in Austin's blistering summer heat. According to John, what Billie lacked in finesse, he made up for in determination, attempting to run down every shot, strategic or not. He didn't want to miss a thing and perhaps that's how he approached life. Billie Turner surely lived his 95 years to the fullest and with the utmost exuberance.

Mark W. Bierner, Ph. D. 1971, B. L. Turner, UT Austin.

I knew Billie Turner for 56 years. I met Billie in 1964 when I was a 17-year-old high school senior taking advanced biology at St. Mark's School of Texas in Dallas. As part of that course, we were required to do a research project. My biology teacher, who had been a Turner student as an undergraduate at The University of Texas, suggested that I do a paper chromatography study of the orchid species in the St. Mark's greenhouse. He then gave me a couple of papers by Alston and Turner on paper chromatography and hybridization in *Baptisia*. If I told you I understood those papers I'd be lyin' like a rug! At any rate, I ran chromatograms on the orchid species, and then my teacher and I took a trip to UT to visit with Professor Turner. He looked at the chromatograms as if he knew what he was looking at (Ralph Alston was, after all, the chemistry guy), and told me that they were basically worthless. Undaunted, I was still excited about the combination of botany and chemistry, and I told Billie that I wanted to get my Ph.D. in biochemical systematics with him as my major professor. After he got finished laughing, he shook my hand and wished me well. I matriculated (that's what we said at St. Mark's) at UT in the fall of 1964, took freshman biology from Billie, got to know Ralph Alston and Tom Mabry, graduated with my B.A. in Botany in 1968, and received my Ph.D. in systematic botany in 1971 with Billie Turner as my major professor. I made good on what I told him as a high school senior. During my undergraduate and graduate years, Billie was my teacher and my mentor. In the years that followed, he was my colleague and friend. I loved him dearly, and I miss him terribly. May his memory be a blessing for us all.

David Northington, Ph.D., 1971, B. L. Turner, UT Austin. (1967-1971).

In 1967, I was a newly accepted candidate to work toward my graduate degree in Botany under the guidance of Billie Lee Turner as my major professor. That next Spring (as best I can remember) there was an uprising by UT students protesting the rerouting of Waller Creek on campus near the football stadium. This rerouting was planned to allow the stadium to add an upper deck on the west side. On the day the bulldozers were to remove several old live oaks, dozens of ecologically minded students tried to block the dozers. This strategy was handled fairly easily by campus police. However, a few enterprising protesters managed to zig zag through the police and climb up fairly high into a couple of trees. One of the Regents present ordered the dozers to go ahead and clear the trees with the students in them! (Cooler heads decided that was not really a good idea so the dozer drivers clocked out early). The "Daily Texan" (school newspaper) had a photographer covering the event so the next morning there was an article complete with a picture of me fairly high up a tree. From that picture the Dean of the College found out the name of the student and then found out he was in graduate school and was a student of Dr B. L. Turner - who was told the Regent in question wants that student kicked out of school, immediately! Short story, somehow Billie talked the Dean into not kicking me out of school if Billie could convince me to reconsider my career choices and focus on a herbaceous genus instead of a woody one. Good advice! I did.

A few years later after I graduated and attained a tenured Assoc. Professor in the Department of Biological Sciences at Texas Tech University, I ventured on a week-long field trip to Oaxaca (mentioned by Wayne Ellison above), which included me, Billie, Wayne and another UT graduate student just starting an MS program. The trip was not totally a spur of the moment idea, as Billie and I had talked about collecting that area for several months previous. I volunteered to check out a vehicle from Texas Tech's motor pool and do most of the driving - so I rolled in to the parking lot East of the UT Biology building to meet up with Billie and meet Wayne and the other grad student (apologies for not remembering their name). The UT crew had ample plant presses, snacks, water bottles, maps, etc. including a small piece of luggage to allow for changes of clothes and toiletries - except Billie who had a smallish paper bag of a tooth brush and a few items of apparel. As Wayne has mentioned, we collected a substantial amount of taxa that had not previously been described - so a successful botanical collecting trip - which included a couple of non-botanical events. On one of the days heading down the Oaxaca highway, a roadblock manned by four men dressed in typical Kakhi uniforms, rifles and a sedan parked at an angle on the side of the road appeared ahead, so I started to slow down a bit. Billie looked up from the back seat and said to wait until he gave me the word and then to floor the gas pedal and absolutely do not stop! About 50 yards from the roadblock, Billie shouted "hit it!!" - or some similar suggestion - which I did and blew through the roadblock gaining speed toward 85 mph. As realistic as the men and guns looked, Billie noticed that their sedan was certainly not a police car - it was an attempt of bandidos to stop us and rob us! Apparently, Billie had been around that block previously. Having burned a lot of adrenaline induced energy, later that day we decided to look for a motel for the night. Our first stop was a collection of separate one room units that had three-sided carports on the back side. Billie went up to the office to secure our lodging for the night - but when he came back he said they rented only by the hour, not by the night. We went on down the road to find more conventional lodging. Oh, the curious choice of Billie's "luggage" that held only a few items of clothing was clarified early on the trip as he shed his shorts and shirt to keep them clean - within the first hour of leaving campus (as I recall). Wayne probably has greater clarity about the timing - as he shared the back seat with Billie for the entire week. I do remember suggesting to Billie that he should get dressed before we came to the Mexican border. He did.

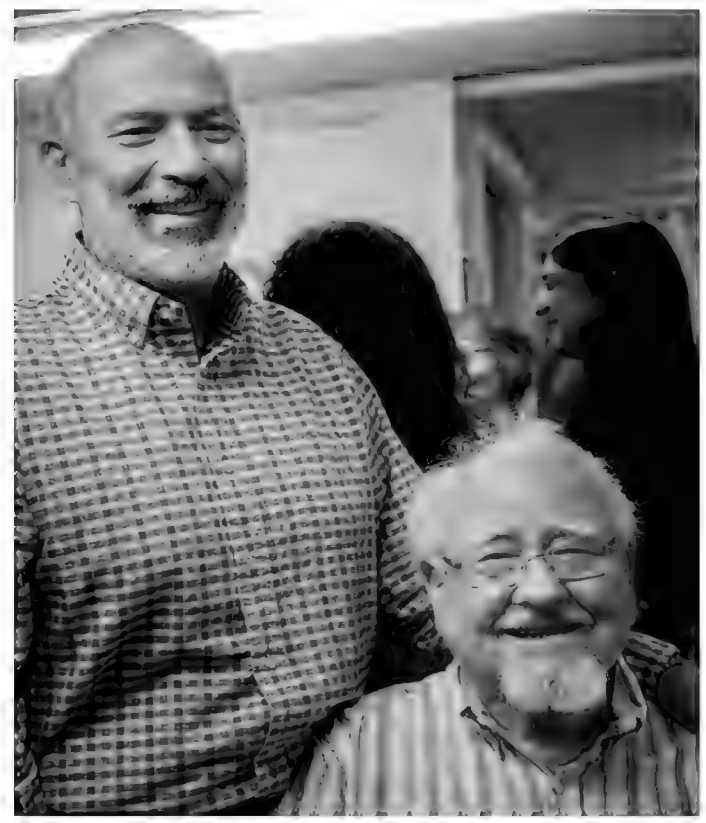
Susan Plettman Rankin, Partners 1975-1979

I remember spending time with Billie in Glacier National Park. The joy with which he pointed out the emerging glacier lilies, expanses of fireweed, puffs of bear grass, and then the little Saxifragaceae, "delicate and beautiful" he said in his happy lilt, was a wonder of his joy in the field. And I recall his comment about how he would have leapt down the snowy steep wildflower-covered slope only to save his son Matt. He was always thinking, and talking, at speed and his mind was always going.

And, of course a comp memory- We were botanizing in the Everglades driving along a bladed shell road. As always, Billie spotted a comp and said "will you please hop out and collect that ___ (comp)?" I jumped out of the car, and over the hump of bladed shell and was headed toward the comp. Billie then said in a calm and slow voice "STOP, you just stepped over a BIG rattlesnake." I looked down and indeed right next to my foot was a huge Eastern diamondback rattlesnake, as big as my leg. She was lying out sunning, not coiled. Of course, we all hear to freeze when we encounter a rattlesnake, but nobody says what to do next. I eventually circled back to the car anxiously scanning for the snake's mate. About that time, a swamp geezer pulled up in an old station wagon and said - do you want that snake? We said, no, you can have him. Then Billie and the man proceeded to catch the snake with a snake pole the man had. Then he put him in a cage in his beater. We asked what the man planned to do with it and he replied that he planned to cook it and eat it. The man asked if I wanted to touch him and I said -sure. When the snake wiggled, I realized that Billie and the man had caught it alive. Billie was game to try most anything.

George Yatskievych, Curator, Billie L. Turner Plant Resources Center (TEX-LL), met Billie 1981 University of Texas at Austin

I first met Billie Turner in December 1981, when I was considering the University of Texas for my doctoral studies. Like so many young botanists, I was intimidated to meet the great man, but he put me at ease with his open friendliness and off-color jokes. I went elsewhere for my studies, so did not meet Dr. Turner again until April 2015, when I interviewed for the curatorship of the Plant Resources Center. Even then, I was a bit intimidated, especially as I needed to make a good impression and really wanted the job. Thus, I got to know Billie personally only in his silver years. He was always very kind to my wife and me, and it felt humbling to receive compliments from someone who devoted so much of his career into developing the facility I was hired to care for. Billie still came in daily and was driven to finish work on as many tribes of his Comps of Mexico project as possible, knowing that Father Time was leaning on his shoulder. I have vivid memories of him shuffling slowly through the herbarium using a walker that had a shelf on it where he balanced folders of specimens that he was taking to his office to study. When moving past the cabinets deep in thought, it always seemed as though he had a personal memory to accompany some of the specimen treasures in each case (and maybe he did).



Billie and Dr. Jose Panero, his fellow asterologist, at dedication of the Billie L. Turner Plant Resources Center, 31 Oct. 2017.

When the university decided to rename the Plant Resources Center in his honor, Billie did not seem pleased. By that point in his life, he was a bit shy of further accolades. Shy is not a word normally associated with Billie Turner! He was convinced to attend the small celebration that was held to celebrate the renaming of the Center and cried a bit as friend after friend praised him for his vision and accomplishments.

Billie would become agitated whenever the Colorado botanist, Bill Weber, called the herbarium to speak with him. Dr. Weber, who passed away recently at the age of 100, liked to annoy Billie by pointing out that Billie was a youngster among botanists compared to him. Billie wasn't happy about growing old, but he owned his age and was proud to still be working past his 90th birthday. Yet, when it was time to quit, it was Billie's decision to step away from his "herbarium walker" and to enter full-time retirement. In doing so, he left behind a number of unfinished projects, including undescribed taxonomic novelties, as well as unpublished treatments for the Flora of North America project and a second edition of his Atlas of the Flora of Texas. Billie had a remarkable clarity of vision about such things and when he

decided it was time to retire, he never looked back. Had arthritis not affected his ability to type, we might have expected more juicy memoirs to follow-up his books on his graduate students and his early association with Homer Shantz, or perhaps he finally would have published a collection of his poems. A busy life is never finished, especially when it lives on in so many persons' memories.

Wayne Elisens, Ph. D. 1982, B. L. Turner, UT Austin, 1978-1982.

The genus name that almost was. Based on my dissertation monograph on subtribe Maurandyinae (Plantaginaceae), I was preparing to describe two new genera in 1982. I mentioned to Billie that I would like to honor him, but there was already a genus *Turnera*. Did he have any suggestions for a name? With a smile on his face and a tongue in his cheek Billie suggested the name *Turnerovera*, which I quickly rejected. We both shared a hearty laugh at this novel but not wholly unexpected nomenclatural proposal. With professional aplomb, he then suggested that I honor another individual.

Fieldtrip to Sierra Madre del Sur, Oaxaca. In 1980 I was fortunate to spend a week in the field with Billie, David Northington, and another graduate student. Since the trip entailed hours in a car sitting next to Billie, we both shared many experiences that shaped our lives. What an incredible opportunity to obtain a deeper understanding of the man behind the legends. Suffice it to say that Billie admirably rose above difficult childhood and adolescent circumstances through force of personality and intellect. He has my deepest respect for that aspect of his life. He remembered some of my stories as well, since they turned up in altered form in his memoir "*All my Academic Children*". There also were many botanical highlights of this trip to a biodiverse but poorly botanized region. I'll always recall Billie exclaiming "*new species!*" at several roadside stops in the Sierra after a quick perusal of a plant (comp and non-comp). His knowledge of the Mexican flora was truly remarkable and he described several new species from this collecting foray. Besides new species, enough memories and stories were generated to last a lifetime.

Photos taken in 1980 on the aforementioned field trip in Oaxaca along Mexico route 175, Carretera a Tuxtepec. Billie was never one to miss an opportunity to have some fun, which made the trip enjoyable yet somewhat unpredictable.

In the upper photo, Billie is showing his macho and loco side with a machete found alongside the highway. The bottom photo shows Billie posing with an araceous substitute for a fig leaf hoping to attract a roadside Eve. He was very proud of his petiole.

Randy Scott, Ph. D. 1986, and Tina Ayers, Ph. D. 1986, B. L. Turner, UT Austin, (1981-1986).

Most people's first meeting with Billie Turner are undoubtedly memorable. His office door was open, but I knocked, then entered when he called out from the back. I introduced myself and he asked, "What can I do for you?" When I told him I was a new graduate student of his (later, it became clear that there were a number of us who came to study with him that year, so he can be forgiven), he asked questions, many questions, as I had been warned he would by certain older grad students, but one that stands out was whether I was married or had a girlfriend. When I said that I wasn't married but had a girlfriend who would be joining me in December, he quickly said that, no, she wouldn't come, but not to worry, he had a wonderful woman in mind for me. As might be guessed, the girlfriend stayed in Montana and, after a year of avoiding each other, in spite of Billie's cajoling, the woman Billie "had in mind" and I went out for dinner and we are still together. Two or three years later, Billie, Tina and I went to northern Mexico to collect plants. Nearly on a whim, he introduced us to the Hintons at their family compound south of Saltillo where we spent the afternoon chatting with them while watching innumerable Monarch butterflies that had momentarily taken up residence there. We would spend a few more days collecting before we ended up in Monterrey where Billie paid for a large suite at the luxurious Hotel Chipinque overlooking the city. Early the next morning, Billie slipped into our room, jumped on our bed, and said, in his inimical way, "There. You can tell people you woke up in bed with Billie Turner!" With that, he turned and left. Afraid that we would never get married, he "married" (his term) us with the specific epithet "ayerscottiana." There are numerous stories we could tell of Billie's generosity not only financially, but in many other ways during both our graduate careers and afterwards. Over the years, we would visit not only in Austin, but, also, at his house in Bigfork, MT and, later, in Alpine. In every setting, he was always Billie, welcoming, questioning, feisty. Never a dull moment.

Matt Lavin, Ph. D. 1987, B. L. Turner, UT Austin.

Billie loved his academic children. Billie expressed this when I was his PhD student, 1983-1987, by the way he reviewed our proposals and manuscripts, hosted graduate student parties, occasionally took us to lunch at the faculty club, helped fund our collecting trips to Mexico, wrote us highly supportive reference letters for job applications, attended celebrations of our newborns, and carried on in a positive manner while encouraging our scholarly independence. His love was evident also by the way he maintained contact after his academic children departed Austin. In my case, post-Austin contact started out with a summer visit to Bozeman when Billie and family were making their way up to Big Fork, Montana, and ultimately settled on just a love note from Billie, which was penned on the back of the holiday card we had just sent him. The exception to this latter routine was when my daughter, Amanda, visited her friends in Austin in 2015, which was about 30 years after her Austin birth. Amanda had the good fortune of having lunch with Billie and Matt at the faculty club. For a brief time, Amanda's visit rejuvenated my communication with Billie. This included a phone message (I still have it on my phone), a couple of phone calls, and a photograph. In these exchanges, Billie conveyed his satisfaction of having a fine relationship with not only his academic children but also with at least one his academic grandchildren of sorts, Amanda. Billie's expression of love for his academic children, although intermittent, remained highly palpable even after 30 years from the last time I saw him.

Billie and Matt Turner with Amanda Lavin during lunch at the faculty club, 9 July 2015.

Richard Olmstead, Ph.D. 1988, University of Washington, Student of Billie Turner: 1971 (U Montana Field Biology Station), 1973-74 (grad student at UT; no degree earned);

My undergrad curriculum required that all biology majors spend a summer at a field biology station. Our school maintained a good one at a beautiful location in the Adirondacks, staffed and attended by faculty and students from the College, but I was counseled by a botany professor, instead, to go to the Montana field biology station on Flathead Lake, where students came from around the country to take classes from prominent professors from many institutions. It was a fateful choice; a Texan named Billie Turner taught the Plant Taxonomy class the summer of 1971. Three days a week for 8 weeks of Billie – lecturing, in the field, in the lab – what an entertaining summer! Two years later I started grad school with Billie. A classmate of mine also attended that summer; Tom Wendt started at Texas the year before. When I arrived, beard, long hair, VW microbus, and all, I was assigned an office with the other 'hippie' in the department, Michael Dillon. While Tom and Mike went on to careers recognized for their work on the Peruvian and Mexican floras, respectively, I washed out. It was neither the time nor place for me to be in grad school. I don't know how many UT professors told me to "Stay and salvage a Masters," or "If you quit here, don't ever expect to get back into grad school anywhere," or things that implied that I just didn't have what it takes to do a Ph.D. How right they were – then. Billie said: "Follow your heart and when you're ready to go back, get in touch." Ten years later the time was right and I got back in touch with Billie. I applied to three schools that year and Billie wrote letters on my behalf. He didn't just write letters of recommendation to the institutions, he reached out personally to the faculty with whom I might study at each institution where I had applied to explain why they should give me a second chance. I don't think I would have succeeded without those personal contacts. He had no reason besides the love he had for his students to do that for me.

I was a student at UT for a year and a half and never identified a thesis project, but I remember one conversation with Billie in which I said I was interested in how families of plants were related to each other. Billie said that if I wanted to contribute in that area I should start by working on a genus and learn how species are related, then look at the genera in a family, etc. and by the time I was his age (~50), I might have something to contribute. Years later, when I was a postdoc and sequencing DNA, something Billie predicted in his 1969 essay, I published one of my first papers on the relationships among families in Asteridae.

Fred Barrie, Ph.D. 1990, B. L. Turner, UT Austin, currently at Missouri Botanical Garden, 4344 Shaw Blvd. St. Louis, MO 63110.

When Amy Jean Gilmartin, my advisor for a Masters at Washington State, learned that I was headed to Austin to study with Billie Turner, she smiled, chuckled and shook her head, thinking of the experience I had in store. She thought we'd get along well. Arriving in Texas, I met an energetic man with a charming grin, a tremendous enthusiasm for botany, and a penchant for asking questions so personal that they would embarrass a therapist. Billie could get away with doing that, in a way that no one else I've ever encountered could.

Billie was not a helicopter advisor. He did not hover over his students. Although in the 1980's, when I was there, his focus was on Mexican Asteraceae, he was happy to let his students work on whichever group piqued their interest. We were free to design our own research programs and independent work was encouraged. Whenever there was a question, however, or when an issue of any kind arose, he was always available, generous with his knowledge and insights. He was committed to education, not only of graduate students but undergraduates as well. He made a point of teaching freshman biology every year, committed to exposing the students to the evolutionary concepts that, if they came out of Texas public schools, he believed they had never before encountered.

In the fall of 1984, or possibly 1985, Billie spent a term in residence in Xalapa, Veracruz. That year I was collecting extensively in Mexico and right around Thanksgiving I found myself in Xalapa, where Billie put me up for the better part of a week. One hot and muggy day we went collecting on Mt. Orizaba. After we finished, we stopped in a restaurant. It was a nice place, more upscale than most I had been frequenting on that trip. We sat down and the waiter brought us menus and two big glasses of ice

water. As anyone who has travelled in the tropics knows, drinking ice water in an unfamiliar restaurant is ill-advised. But I was hot, tired and thirsty and, against my better judgement, I grabbed that big glass and started drinking it down. Billie was sitting opposite me and, as the water level got lower and lower in the glass, Billie's eyes opened wider and wider, and is jaw dropped closer and closer to the tabletop. When I slapped the glass back on the table, Billie was staring in stunned amazement. It was the only time I can remember seeing Billie speechless. I paid for my recklessness the next day, but it was worth it for the look on Billie's face.

Piero G. Delprete, Ph.D, 1996, B. L. Turner, UT Austin, 1990-1996

Christmas presents. Billie studied mostly the flora of Mexico and of Texas. He had a special relationship with the Hinton family, based in Mexico, whose several generations collected plants in numerous localities in that country. While I was a doctoral student at UT Austin, boxes of dried specimens collected by the Hintons arrived regularly at the herbarium. A quick search in the databases available in the internet results in 52 specific epithets that Billie dedicated to members of the Hinton family, in genera of the Asteraceae, Amaryllidaceae, Boraginaceae, Nyctaginaceae, Caryophyllaceae, Papaveraceae, Rubiaceae, Commelinaceae, Fabaceae, Gentianaceae, Campanulaceae, Polemoniaceae, Oleaceae, Loasaceae, Hydrophyllaceae, Lentibulariaceae, Crassulaceae, and Melianthaceae. As soon as a Hinton's box arrived, he and Guy Nesom, the herbarium curator at that time, ran to it with the same excitement of two kids discovering their new Christmas present. As they were going through the specimens in the box, they would immediately recognize the new undescribed species, because of their knowledge of the flora of Mexico. One of them would say "This is mine" and the other "No! This is mine! Oh well, ok, this is yours, but the next one is mine." And so on, until both of them had a bundle of specimens of new species to describe. The next day, after careful study of pertinent literature and herbarium specimens, they started writing their manuscripts. By the evening of the same day they had a few manuscripts finished, and they exchanged them with each other for checking the texts before submitting them. Billie often returned the manuscripts to Guy with the note "clean as a cat's ass!" Billie often asked me to make the accompanying line drawings. At that time I had no experience with botanical line drawings. So, I bought technical pens and paper, and started to produce some drawings. The first ones were not very good. But after some practice, they became acceptable. And as the drawings were finished, the manuscripts were submitted to *Phytologia*.

Work ethic. I never had the occasion to accompany Billie in field trips for botanical collections. The only excursions in natural vegetation with him were those made as teaching assistant for his field course "Plants of Texas" at the Brackenridge Field Station of UT Austin, along with groups of students. The real learning experience for me was working side by side with him in the herbarium. We shared the same bench for more than five years. What most impressed me was his work ethic and his keen capacity of observing morphological details, which allowed him to discriminate morphological differences used to describe new species. He loved what he was doing. Botany was his main entertainment. He arrived at the herbarium at 8 am every day, including many Saturdays and Sundays, and would leave at 5-6 pm. We exchanged morphological observations and discussed just about any topic, while looking through our dissecting microscopes. He was generally a very loving and entertaining person, and always very direct when he had things to say to anyone. One day, a box arrived that contained specimens that have been on loan to another herbarium for several years. He noticed that none of the specimen were annotated by the supposed specialist. He immediately picked up the phone and called the curator of the other herbarium. After a conversation where the volume of the two curators was raised a few times, he concluded the discussion with "there is only one reason for not annotating specimens that were received on loan: death!"

Karen Clary, Ph.D. 1997, B. L. Turner, UT Austin.

Billie led a long and amazing life and was an academic father to so many UT botanists. I can't think of any adjective or even list of adjectives that would fully describe Billie Lee Turner. He was so many things and truly bigger than life. I have many memories of Billie from my days at UT Botany and beyond. I spent a lot of time in the UT herbarium. Billie loved to talk and would tell me many a tale about

his life and botany, which I came to realize were one and the same for him. He named a little sunflower (*Senecio claryae*) that grows on the gypsum slopes of Cerro Alto in Coahuila in my honor. Billie, Tom Patterson and I had found it while botanizing the area in 1993. Billie, a most prolific collector of *Senecio*, had already named another *Senecio* after Patterson, so he decided to name it after me.

Nuclear and chloroplast DNAs reveal diverse origins and mis-identifications of *Juniperus chinensis* cultivars from Windsor Gardens, UK. Part 2 of 3.

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ABSTRACT

Ploidy was determined for twenty four (24) plants labeled as *Juniperus chinensis* cultivars at the Windsor Gardens, UK and revealed 16 were tetraploids (2*n*=4*x*=44), 7 diploids (2*n*=2*x*=22), and one triploid (2*n*=3*x*=33). nrDNA (ITS) and cp DNA sequencing found one of the diploids was actually a cypress; *J. chinensis* cv Savill Sentinel was *Cupressus gigantea*. A second diploid, cv 'Spartan' was *J. virginiana*. Only three of the remaining 22 '*chinensis* cultivars' had both nrDNA and chloroplasts (cp) of *J. chinensis*: Iowa (=Globosa), Obelisk and Plumosa Aurea and these, having homozygous nrDNA, appear to be autotetraploids. Four other cultivars had *J. chinensis* nrDNA but cp of *J. tsukusiensis*. Two cultivars, Richeson and Fruitlandii, were determined to be *J. xpfitzeriana*. Two cultivars, Japonica and Japonica Variegata, had nrDNA and cp of *J. chinensis* var. *sargentii*. The remaining ten '*J. chinensis* cultivars' had *J. chinensis* hybrid nrDNA. But, these 10 cultivars had 3 kinds of cp DNA: 7 had *J. chinensis* var. *sargentii* cp; 2 with *J. sabina* var. *balkanensis* cp; and one, Kek, had *J. chinensis* cp. The amount of hybridization among the parents of cultivars in botanic gardens makes it very difficult to identify cultivated junipers. In this sample of 24 '*J. chinensis*' cultivars, only 3 plants were 'pure, autotetraploid '*J. chinensis*' by DNA. A DNA barcode system, if utilized, would greatly aid botanic gardens to screen current and incoming accessions to assign taxonomic names to junipers. Published on-line www.phytologia.org *Phytologia 102(3): 106-115 (Sept 21, 2020).* ISSN 030319430.
KEY WORDS: *Juniperus chinensis* cultivars, origin, nrDNA, ITS, cp DNA, DNA barcoding.

It has now been shown that genome size assessment using flow cytometry (FC) can be successfully used as a proxy for ploidy level in *Juniperus* (Farhat et al. 2019a, b) from both fresh and silica gel dried leaves of *Juniperus*. Thus, the ploidy of Juniper hybrids can now be determined by FC. This is very important because it is known that several *J. chinensis* cultivars are triploid or tetraploid (Hall, et al. 1979). With the confluence of both DNA methodology and FC ploidy determination, this presents us with a great opportunity to examine the origin of *J. chinensis* cultivars.

As a first step in this work, we recently analyzed *Juniperus* x*pfitzeriana* cultivars, one of the most commonly cultivated junipers in the world (Adams, et al. 2019). The origin of *J.* x*pfitzeriana* is thought to be a hybrid of *J. chinensis* x *J. sabina*. Nuclear DNA (nrDNA, ITS) and 4 chloroplast gene regions were sequenced from 14 *J.* x*pfitzeriana* cultivars from Windsor Gardens, UK, and compared with all *Juniperus*, sect. *Sabina*, smooth leaf margin species. All of the 14 cultivars were identical in their chloroplast DNA and their cp DNA was identical to that of *J. sabina* var. *balkanensis* (Table 1). In addition, 13 *J.* x*pfitzeriana* cultivars were allo-tetraploids with heterozygous bases at 5 to7 sites that distinguish *J. chinensis* and *J. sabina* var. *balkanensis*. These cultivars had identical nrDNA. Two of the 14 cultivars, 'Old Gold' and 'Sea Green', showed a slightly different nrDNA pattern, being homozygous at sites 410 and 1139, as found in *J. s.* var. *balkanensis*. The origin of *J.* x*pfitzeriana* is from a cross of a male, tetraploid *J. sabina* var. *balkanensis* and a female, tetraploid, *J. chinensis*, resulting in an allo-tetraploid, dioecious, *J.* x*pfitzeriana* (Spath) Schmidt.

Table 1. nrDNA (ITS) variable sites in *J. chinensis* cultivars. (Windsor Gardens), *J. chinensis*, and *J. sabina*. K=G/T; S=C/G; Y=C/T; M=A/C; W=A/T; R=A/G. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*/ *J. thurifera*; *sabina* = *J. sabina* var. *sabina*; and *chinensis* = *J. chinensis*. Modified from Adams et al. (2019). Site numbers modified to correspond with site numbers in Table 3 of this report.

taxa: *J.* x*pfitzeriana* (=x*media*), unless noted otherwise	ploidy	212[a] K	410 S	665 Y	986 Y	996 M	1034 K	1073 W	1137 R	ITS classification hybrid?	chloroplast, ex. pollen from:
Most probable male (pollen) parent	4*x*	G	C	T	T	A	T	T	G	*J. sabina* var. *balkanensis*	*J. sabina* var. *balkanensis*
Most probable female parent genotype	4*x*	T	G	C	C	C	G	A	A	*chinensis*	*chinensis*
15442 Arctic	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15454 Armstrongii	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15418 Aurea, Paris-sud	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15474 Aurea	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15423 Saybrook Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15425 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15463 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15443 Gold Star	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15462 Golden Saucer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15482 Goldenkissen	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15430 pfitzeriana prostate	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15453 Old Gold	4*x*	G/T	**C**	C/T	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15436 Sea Green, Windsor	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15604 Sea Green Home Depot, Inc.	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis?*

[a]Variable sites located at: 212, xGGCCAAGC; 410, xGTTGAGAT; 665, xTCTTCGTC; 986, xGCCCTCCC; 996, xGCGAGGAG; 1034, xGCGGTCGG; 1073, xCGCGACGA; 1137, xGAACTTTG.

The purpose of the present research is to present new DNA sequencing utilizing both chloroplast and nuclear DNA in the determination of the origin of *J. chinensis* cultivars.

METHODS

Plant materials:

Samples: Leaf samples were collected in Windsor Gardens, Windsor Great Park, Windsor, *SL4 2HT* UK from 24 *J. chinensis* cultivar accessions (see Table 2) and immediately placed in activated silica gel for DNA sequencing and Flow Cytometry - ploidy determination.

Table 2. Windsor 24 *Juniperus chinensis* cv and origin table < = earlier than (before).

taxon *Juniperus chinensis* '	Adams #	Windsor acc. #	ploidy this study	Chrom number, 2*n*, litr.	Origin: based on Den Oden and Boom 1965; Krussmann 1991; Welch 2012, Lewis 1998, Auders & Spicer 2012.
J. chinensis 'Savill Sentinel'	15426	2003-153	2x		1999, cutting ex *J. chinensis* (1999-6117), Windsor Card
J. chinensis "Shepherdii'	15471	1999-757	4x		China (Robert Fortune) 1855 but named in 1867
J. chinensis ' 'Belvedere' ,='Armstrongii'	15427	2000-271	3x	(44)	'Belvedere' Austria 1973; 'Armstrongii' Canada 1932
J. chinensis ' 'Keteleerii'	15432	1999-5819	4x	(44)	Belgium <1910
J. chinensis ' 'Japonica'	15433	2001-465	4x		1855 Carriere
J. chinensis 'Japonica Variegata'	15439	1999-5816	4x	(44)	1867 Carriere
J. chinensis 'Kuriwao Mist'	15441	1999-5821	4x		New Zealand < 1993?
J. chinensis 'Kuriwao Sunbeam'	15446	1999-5822	2x		New Zealand <1993
J. chinensis 'Richeson' = x pfitzer	15451	1999-5832	4x		= x pfitzer USA 1941, pfitzer sport
J. chinensis 'sargentii 'Glauca'	15452	1999-5996	2x	(22)	UK 1855
J. chinensis 'Lombarts'	15458	2000-1334	4x		Windsor Great Park <1998?
J. chinensis 'Aurea' = 'Alba'.	15461	1999-5805	4x	(44)	'Aurea' 1855 UK; 'Alba' = 'Plumosa Albovariegata'
J. chinensis 'Spartan'	15464	1999-5838	2x		USA 1950s
J. chinensis 'Jacobiana'	15466	1999-6183	4x	(33)	< 1887 = 'Hetzii'
J. chinensis Pfitzer Gp. 'Blaauw'	15466	1999-6078	2x	(44)	Japan, Introduced by Blaauw & Co., 1924, Netherlands
J. chinensis 'Robusta Glauca'	15467	1999-5833	4x		unknown
J. chinensis 'Obelisk'	15469	1999-5829	4x	(44)	Japan seed germinated in Holland 1930
J. chinensis 'Iowa' = 'Globosa'	15470	1999-5814	4x	(44) (22?)	USA 1930
J. chinensis s 'Fruitlandii'	15472	1999-5812	4x	(33)	x media =x pfitzer USA 1977
J. chinensis Pfitzer Gp. 'Shimpaku'	15473	1999-6111	2x		= x pfitzer, Japan <1966
J. chinensis Pfitzer Gp. 'Globosa Cinerea'	15477	1999-6083	2x	(44) ?	Japan <1930
J. chinensis Pfitzer Gp. 'Plumosa Aurea'	15478	1999-6105	4x		<1884
J. chinensis 'Kek'	15484	1999-5818	4x		Windsor Great Park 1992?
J. chinensis 'Mathot'	15488	1999-5826	4x		Holland <1947

Reference Species: *Juniperus chinensis*, *J. sabina* var. *sabina*, *J. s*. var. *balkanensis* see Adams et al. (2018a) for collection details.

DNA extraction and sequencing

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Chromatograms analyzed by use of Chromas 2.31 (Technelysium Pty Ltd.).

Flow cytometric analyses for ploidy level determination

Nuclear DNA amount was assessed by flow cytometry (FC) based on the technique of Bourge et al. (2018) on silica dried leaves of *Juniperus* samples and fresh leaves of *Hordeum vulgare* L. 'Sultan'[2C= 9.81 pg in Garnatje et al. (2004)] used as an internal standard. Approximately, 30 mg of leaves of both the internal standard and *Juniperus* were simultaneously chopped using a razor blade in a plastic Petri dish with 500 µl of cold Gif nuclear-isolation buffer-GNB (Bourge et al. 2018): 30 mM sodium citrate, 45 mM $MgCl_2$, 60 mM MOPS (4-morpholine propane sulphonate, pH 7), and 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2 containing 0.1% (w/v) Triton X–100, supplemented with 10 mM sodium metabisulphite and RNase (2.5 U/ml). The nuclei suspension was filtered through 50 µm nylon mesh. The nuclei were stained with 100 µg/ml propidium iodide (PI), a specific DNA fluorochrome intercalating dye, and kept at 4°C for 5 min. DNA content of about 3,000 stained nuclei was determined for each sample using the cytometer CytoFLEX S (Beckman Coulter- Life Science United States. Excitation 488 nm, 26 mW; emission through a 610/20 nm band-pass filter). Measurements of each sample were repeated twice. The software CytExpert was used for histogram analyses. The total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the species and the internal standard, according to the following formula:
2C DNA sample (pg) = (Sample 2C peak mean / Standard 2C peak mean) x Standard 2C DNA (pg).

RESULTS AND DISCUSSION

Ploidy was determined for twenty four (24, only 23 were *Juniperus*, see below) plants labeled as *J. chinensis* cultivars at the Windsor Gardens, UK and analyses revealed (Tables 2, 3) that of the 23 juniper plants, 16 were tetraploids (44), 6 diploids (22), and one triploid (33). Farhat et al. (2019a) discovered that about 15% of *Juniperus* taxa were tetraploids and one, *J. foetidissima*, was a hexaploid, based on analysis of samples from junipers that were naturally occurring not cultivated. In this study, we found most of these cultivated plants were tetraploids. It is worthwhile to review an interesting study by Zinnai and Chiba (1951) who in a survey *Cryptomeria japonica* in seedling nurseries (2 and 3-year old seedlings) found 4 seedlings with twisted needles that were thick and bent at the tip-end. In addition, the stomatal bands tended to be larger. Chromosome counts on these plants confirmed they were tetraploids. Chiba (1951), later, selected 39 (putative) polyploid seedlings with twisted needles from the germination beds and found 18 were diploids, 3 triploids and 18 tetraploids. The polyploids randomly occurred in beds at a rate of 5×10^{-6} frequency (e.g., 0.0005%). Normally in a forest seedling nursery, abnormal appearing seedlings (such as these with twisted needles) are removed by gardeners to maintain robust seedlings for out-planting. Ahuja (2005) noted that "sporadic polyploids and aneuploids occur at a very low frequency in nurseries in conifers, but most of them show growth abnormalities, remain dwarf, and may not reach maturity".

Ploidy shown in Table 2 is compared with literature reports of chromosome number (Hall, et al. 1979). Note that several literature reports differ from the flow cytometry ploidy determination: Belvedere, litr. = tetraploid (44) vs. triploid (33); Jacobiana, litr = triploid (33) vs. tetraploid (44); Blaauw, litr = tetraploid (44) vs. diploid (22), Fruitlandii, litr. = triploid (33) vs tetraploid (44); Globosa Cinerea, litr = tetraploid (44?) vs. diploid (22). It is very likely that there have been labeling errors over the decades in transferring plants among botanic gardens and nurseries. It nearly impossible to obtain samples from the original plants for which the names originated.

Analysis of nrDNA (ITS) revealed 12- 14 polymorphic sites among the 24 'J. chinensis cv' studied (Table 3). Analysis of 3 chloroplast (cp) genes: petN-psbM, trnS-trnG and trnL-trnF revealed that petN-psbM (hereafter petN), as the most informative in distinguishing *J. chinensis*, *J. sabina*, and related species, thus, trnS-trnG and trnL-trnF were not further utilized. petN sequence utilized to reveal the chloroplast source (e.g., pollen, paternal) for the *J. chinensis* cultivars studied.

The 24 '*J. chinensis* cultivars' were found to be in 8 groups (Table 3). The first group (yellow) included 'Richeson' and 'Fruitlandii, both tetraploids, which have *J. sabina* var. *balkanensis* cp, and *J. xpfitzeriana* ITS, as seen in the Wilhelm Pfitzer (*xpfitzeriana*) sample (from Adams et al. 2019). So, both of these are *xpfitzeriana*, not *J. chinensis*.

Japonica, and Japonica Variegata (2nd group, blue), tetraploids, are part of *J. chinensis* var. *sargentii* (Table 3) with *J. c.* var. *sargentii* cp and ITS.

Kuriwao Sunbeam is in the 3rd (purple) group and is very unusual being a diploid with *J. sabina* var. *balkanensis* cp and *J. chinensis* var. *procumbens* ITS, because both of these taxa are tetraploid (Farhat et al. 2019a).

The 4th and 5th groups are closely related with all 7 cultivars having *J. chinensis* ITS DNA, but the red group 4, contains Obelisk, Iowa (=Globosa), and Plumosa Aurea which are tetraploids with *J. chinensis* cp. In contrast, group 5 (salmon) contains 4 diploids (*chinensis* var. *sargentii* Glauca, Pfitzer Blaauw, Pfitzer Shimpaku, and Pfitzer Globose Cinerea), all have *J. chinensis* ITS, but each has *J. tsukusiensis* (sometimes treated as *J. chinensis* var. *tsukusiensis*, Adams 2014) chloroplasts. The use of Pfitzer as part of the cultivar name is confusing, as *xpfitzeriana* is tetraploid and of hybrid origin from *J. sabina* x *J. chinensis*, see Adams et al. 2019).

The 6th group (green) is the largest with 9 tetraploids and one triploid, all have *J. chinensis* hybrid ITS DNA (Table 3). Seven (Aurea, Jacobiana, Shepherdii, Keteleerii, Robusta Glauce, Lombards, Belvedere) have *J. chinensis* var. *sargentii* cp. Two (Kuriwao Mist, Mathot) have *J. sabina* var. *balkanensis* cp and one (Kek) is the only plant in these analyses with *J. chinensis* cp. The tremendous diversity in the hybrid nature of nrDNA (ITS) in this group indicating that the maternal parent arose by hybridization with a variety of junipers.

The 9th group was most surprising to find that 'Savill Sentinel' was not a juniper, but a cypress, *Cupressus gigantea* by ITS DNA (Table 3). Interestingly, this plant is of hybrid origin (note the heterozygous ITS sites, Table 3), with a male *Cupressus gigantea* parent chloroplast. We not able to identify the maternal parent of the hybrid at this point. Even with 3 botanists collecting samples, none of us noted that it was a cypress. Perhaps we were too focused on the mechanics of collecting and accurately labeling the samples to observe the plant.

Group 10 produced the second surprise in that 'Spartan' had ITS and cp DNA of *J. virginiana* (Table 3). *Juniperus chinensis* and *J. virginiana* look very similar, especially if juvenile (decurrent) leaves are present on *J. virginiana*, so it is not surprising that Spartan was labeled *J. chinensis* as some time in history.

Five diploid cultivars have cp parents that differ from their homozygous maternal parents nrDNA: Kuriwao Sunbeam (*J. sabina* var. *balkanensis*, cp, *J. chinensis* var. *procumbens*, nrDNA); Glauca, Blaauw, Shimpaku, and Globosa Cinerea (all 4 with *J. tsukusiensis* cp and *J. chinensis*, nrDNA). These 5 cultivars with conflicting cp and nrDNA seem likely to have experienced a chloroplasts capture event as has been found often in natural populations of *Juniperus* (Adams et al. 2017 a,b; Adams et al. 2018 a,b; Adams et al. 2020; Farhat et al. 2019 a,b; Hojjati et al. 2019).

It is interesting that some of the aforementioned diversity was discovered Le Duc et al. (1999) by the use of RAPDs (Random Amplified Polymorphic DNAs). Figure 1 shows a PCO based on 122 RAPD bands of *J. chinensis*, *J. sabina* and 9 cultivars. Notice the Pfitzer cultivars group are near the base of *J. chinensis*, but intermediate on axis 2, to *J. sabina*, giving an evidence that they are *chinensis* x *sabina* hybrids, although the synthetic (computer generated) hybrid is precisely intermediate. Fruitlandii (a

xpfitzeriana, Table 3) is intermediate on axis 3. Kallay's Compact, Gold Coast and Hetzii form a group near the Pfitzers (yellow oval).

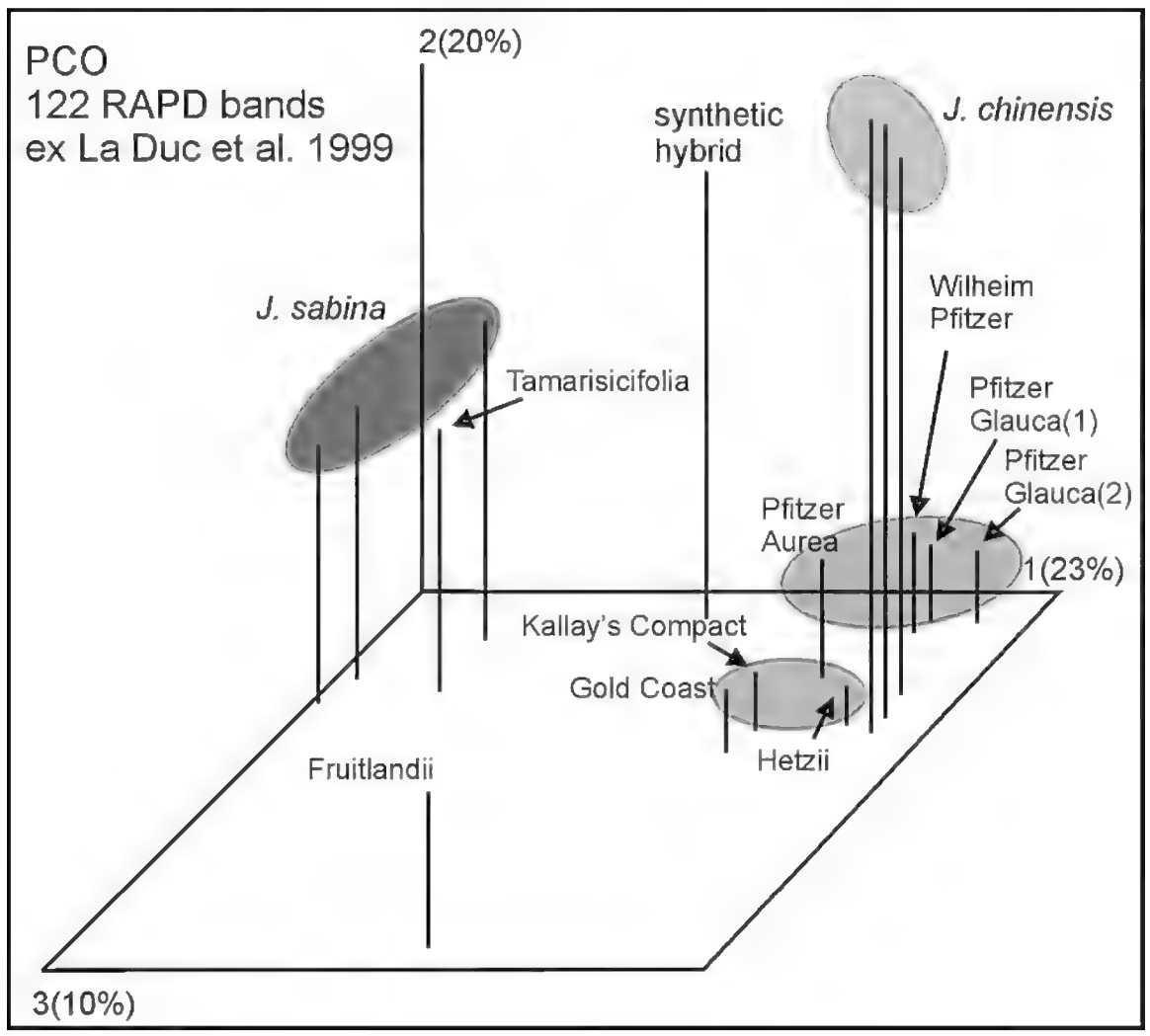

Figure 1. PCO using 122 RAPD bands of *J. chinensis* (natural, Japan), *J. sabina* (natural, Switzerland) and 9 cultivars.

Possible ploidy levels of putative parents of '*J. chinensis*' cultivars in this study

It is very interesting that the ploidy of all the male parents of the '*J. chinensis*' cultivars as well as the female parents have been reported (Farhat et al. 2019a) as tetraploids (4x), (Table 4: *J. sabina* var. *balkanensis*, *J. chinensis*, *J. c.* var. *sargentii*, *J. c.* var. *procumbens*, *J. tsukusiensis*, and *J. xpfitzeriana*). However, Kuriwao Sunbeam is diploid (2x, Table 4) suggesting that haploid (1x) gametes of *J. s.* var. *balkanensis* and *J. c.* var. *procumbens* united to form the diploid. The four male *tsukusiensis* x female *chinensis* parentages resulted in diploid (2x) *chinensis* var. *sargentii* 'Glauca', and 3 Pfitzer 'Blaauw', 'Shimpaku', and 'Globosa Cinerea' (Table 4). Although Farhat et al. (2019a) found their natural *tsukusiensis* to be 4x, it is very possible there are cultivars of *tsukusiensis* that are diploid. And, it is certainly possible that putative 'chinensis' female parents were diploids. Unfortunately, we know very little about variation in ploidy of *J. chinensis* in the wild. In a recent study of nearly all *Juniperus* species, Farhat et al. (2019a) reported that *J. chinensis, J. c.* var. *procumbens*, and *J. c.* var. *sargentii* were tetraploids in nature. However, only one plant each of *J. chinensis*, *J. c.* var. *procumbens*, and *J. c.* var. *sargentii* were analyzed. Nagano et al. (2000, 2007) analyzed *J. chinensis* varieties from Japan and reported that *J. chinensis* var. *chinensis*, *J. c.* var. *kaizuka*, *J. c.* var. *jacobiana* were tetraploids (2n=44), but *J. c.* var. *sargentii* was a diploid (2n=22). In Nagano et al. (2007), they report that their *J. c.* var. *sargentii* was obtained from Mt. Shiroiwa, Miyazaki Prefecture. Farhat et al. (2019a) obtained their *J. c.* var. *sargentii* from Mt. Kirigishi, Furano-Ashibetsu Natural Park, Hokkaido. However, Nagano et al. (2007) strongly felt the chromosome karyomorphological differences between their *J. chinensis* var. *chinensis* and *J. c.* var. *sargentii* warranted the recognition of *J. sargentii* at the specific level. In contrast, Adams and Schwarzbach (2013) and Adams et al. (2011) found that their *J. c.* var. *sargentii* (4x) material was in a well-supported clade with *J. chinensis*, supporting its recognition as *J. c.* var. *sargentii*. The confusion may rest on the fact that *J. c.* var. *chinensis* and *J. c.* var. *sargentii* are difficult identify when collecting.

The final unusual case is that of Belvedere, a triploid with male *chinensis* v. *sargentii* (4x, Farhat et al. 2019a; or 2x, Nagano et al. 2007) and female *chinensis* hybrid (4x) (Table 4). If the var. *sargentii* was 2x and the female chinensis hybrid was tetraploid, then the triploid follows simply (2x + 1x = 3x). If the male parent was a tetraploid, then the explanation of triploid hybrid would be more difficult.

Table 4. Analyses of ploidy of putative parents' ploidy and ploidy of the cultivars at Windsor Garden.

Paternal (male) parent cp source	Farhat et al. 2019 ploidy	Maternal (female) parent nrDNA (nuclear) ITS classification	Farhat et al. 2019 ploidy	Windsor Garden accessions grouped by DNA aff. (affiliation): **2 accessions identical to *xpfitzeriana***	Actual ploidy of hybrid
J. sab. v. *balkanensis*	4x	*Juniperus xpfitzeriana*	4x	*15435* Wilhelm Pfitzer *xpfitzeriana*, 4x	4x
J. sab. v. *balkanensis*	4x	*Juniperus xpfitzeriana*	4x	*15451* chinensis 'Richeson' allo-tetraploid = *J. xpfitzeriana*	4x
J. sab. v. *balkanensis*	4x	*Juniperus xpfitzeriana*	4x	*15472* chinensis 'Fruitlandii' allo-tetraploid = *J. xpfitzeriana*	4x
Male parent (cp)	Farhat ploidy	Female parent	Farhat ploidy	***aff: J. chinensis var. sargentii***	Actual ploidy
chinensis/ sargentii[1]	4x	*chin. v. sargentii*	4x	*15433* chin 'Japonica'	4x
chinensis/ sargentii[1]	4x	*chin.* v. *sargentii*	4x	***15439*** chin 'Japonica Variegata'	4x
J. sab. v. *balkanensis*	4x	*chin.* v. *procumbens*	4x	*15446* chin 'Kuriwao Sunbeam'	2x
Male parent (cp)	Farhat ploidy	Female parent	Farhat ploidy	***aff. J. chinensis hybrids***	Actual ploidy
chinensis	4x	*chinensis*	4x	*15469* chin 'Obelisk'	4x
chinensis	4x	*chinensis*	4x	*15470* chin 'Iowa' 'Globosa'	4x
chinensis	4x	*chinensis*	4x	*15478* chin Pfitzer 'Plumosa Aurea'	4x
Male parent (cp)	likely ploidy	Female parent	likely ploidy	***aff. J. chinensis x J. tsukusiensis hybrids***	Actual ploidy
tsukusiensis Farhat 4x	2x	*chinensis,* cultivar?	2x	*15452* chin sargentii 'Glauca'	2x
tsukusiensis Farhat 4x	2x	*chinensis,* cultivar?	2x	*15466* chin Pfitzer 'Blaauw'	2x
tsukusiensis Farhat 4x	2x	*chinensis,* cultivar?	2x	*15473* chin Pfitzer 'Shimpaku'	2x
tsukusiensis Farhat 4x	2x	*chinensis,* cultivar?	2x	*15477* chin Pfitzer 'Globosa Cinerea'	2x
Male parent (cp)	Farhat ploidy	Female parent	likely ploidy	***aff. J. chin.* var. *sargentii* x *chin* hybrid**	Actual ploidy
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15461* chin 'Aurea'	4x
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15465* chin 'Jacobiana'	4x
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15471* chin 'Shepherdii'	4x
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15432* chin 'Keteleerii' ~= 'Kuriwao Mist'	4x
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15467* chin 'Robusta Glauca'	4x
chinensis v. *sargentii*	4x	*chinensis* hybrid		*15458* chin 'Lombarts'	4x
chinensis v. *sargentii*	,2x	*chinensis* hybrid		*15427* chin 'Belvedere'	
J. sab. v. *balkanensis*	4x	*chinensis* hybrid		*15441* chin 'Kuriwao Mist'	4x
J. sab. v. *balkanensis*	4x	*chinensis* hybrid		*15488* chin 'Mathot'	4x
chinensis	4x	*chinensis* hybrid		*15484* chin 'Kek'	4x
Male parent (cp)	Farhat ploidy	Female parent	Farhat ploidy	**Mis-identified taxa**	Actual ploidy
Cupressus gigantea	2x	*Cupressus gigantea*	2x	*15426 chin* 'Savill Sentinel **ID = *Cupressus gigantea* (hybrid)**	2x
J. virginiana	2x	*J. virginiana*	2x	*15464* chin 'Spartan' **ID = *Juniperus virginiana***	2x

In this study, we found tremendous variation among nrDNA and cp parentage. The development and implementation of a DNA barcode system would greatly aid botanic gardens to screen current and incoming accessions to assign taxonomic names to junipers and other conifers.

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Table 3. Analyses of cp (chloroplast) source and nrDNA (ITS) variable sites in *J. chinensis* cultivars (Windsor Gardens), K=G/T; S=C/G; Y=C/T; M=A/C; W=A/T; R=A/G.

Juniperus x pfitzeriana	ploidy	cp source	ITS classif.	Heterozygous sites												
15435 Wilhelm Pfitzer *xpfitzeriana* (ex. Adams et al. 2019)	4*x*	*J. sabina* v. *balkanensis*	*Juniperus xpfitzeriana*	212K	410S	665Y	986Y	996M	1034K	1073W	1137R	Note: nrDNA (ITS) shows it is hybrid *J. sabina* v. *balkanensis* x *J. chinensis*				
15451 chinensis 'Richeson' allo-tetraploid = *J. xpfitzeriana*	4*x*	*J. sabina* v. *balkanensis*	*Juniperus xpfitzeriana*	212K	410S	665Y	986Y	996M	1034K	1073W	1137R					
15472 chinensis 'Fruitlandii' allo-tetraploid = *J.x pfitzeriana*	4*x*	*J. sabina* v. *balkanensis*	*Juniperus xpfitzeriana*	212K	410S	665Y	986Y	996M	1034K	1073W	1137R					
aff: J. chinensis var. sargentii	ploidy	cp source	ITS classif.	Heterozygous sites												
15433 chin 'Japonica'	4*x*	*chinensis/ sargentii*[1]	*chinensis v. sargentii*	301R	638R		[1] ITS ~= var. *chinensis* and var. *sargentii*.									
***15439* chin 'Japonica ' Variegata'**	4*x*	*chinensis/ sargentii*[1]	*chinensis* v. *sargentii*	301R	638R											
15446 chin 'Kuriwao Sunbeam'	2*x*	*J. sabina* v. *balkanensis*	*chinensis* v. *procumbens*[2]	212K			[2]ITS = (100%) to *J. chinensis* var. *procumbens*.									
Juniperus chinensis/ tsukusiensis	ploidy	cp source	ITS classif.	Heterozygous sites												
15469 chin 'Obelisk'	4*x*	*chinensis*	*chinensis*	212R		392S										
15470 chin 'Iowa' 'Globosa'	4*x*	*chinensis*	*chinensis*													
15478 chin Pfitzer 'Plumosa Aurea'	4*x*	*chinensis*	*chinensis*						960Y							
15452 chin sargentii 'Glauca'	2*x*	*tsukusiensis*	*chinensis*		315K											
15466 chin Pfitzer 'Blaauw'	2*x*	*tsukusiensis*	*chinensis*					533K								
15473 chin Pfitzer 'Shimpaku'	2*x*	*tsukusiensis*	*chinensis*		315K					1026R						
15477 chin Pfitzer 'Globosa Cinerea'	2*x*	*tsukusiensis*	*chinensis*				396Y					?				
J. chinensis hybrids	ploidy	cp source	ITS classif.	Heterozygous sites												
15461 chin 'Aurea'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid		212K		350R		431R					1093Y		11489Y
15465 chin 'Jacobiana'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid				351Y							1094S	1137R	1149Y
15471 chin 'Shepherdii'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid	201K 202R		301R										
15432 chin 'Keteleerii' ~= 'Kuriwao Mist'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid	179Y	212K	238Y 230M	351Y 361R	389S	428Y	614Y 615S	883Y	986Y	1073W		1145R	1149W
15467 chin 'Robusta Glauca'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid	201K	212K		351Y	389S	531S	545K 552R	639Y	784Y	986Y	1034R	1035K	1192M
15458 chin 'Lombarts'	4*x*	*chinensis* v. *sargentii*	*chinensis* hybrid	201K 202R	212K		351Y	389S		545K 552R	639Y	784Y	986Y		1035K	1192M
15427 chin 'Belvedere'	3*x*	*chinensis* v. *sargentii*	*chinensis* hybrid		212K		351Y	389S		606M	665Y	784Y	986Y		1073M	1149W
15441 chin 'Kuriwao Mist'	4*x*	*J. sabina* v. *balkanensis*	*chinensis* hybrid	179Y	212K	238Y	351Y 361R		428Y			986Y	1073W	1137R	1145R	1149W
15488 chin 'Mathot'	4*x*	*J. sabina* v. *balkanensis*	*chinensis* hybrid		212K							986Y	996M	1035K	1073W	
15484 chin 'Kek'	4*x*	*chinensis*	*chinensis* hybrid	201K 202R	212K		350R 351Y	389M				986Y			1073W	
15426 chin 'Savill Sentinel **ID = *Cupressus gigantea* (hybrid)***	2*x*	*Cupressus gigantea x ?*	*Cupressus gigantea*	289K	344Y	387R	467R	794K	977M	979R	1179Y	794K				
15464 chin 'Spartan' **ID = *Juniperus virginiana***	2*x*	*J. virginiana*	*J. virginiana*	742K	1019K											

Rumicastrum Ulbrich (Montiaceae): a *beautiful* name for the Australian calandrinias

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ABSTRACT:

For more than 30 years, Montiaceae specialists have agreed that Australian species classified in *Calandrinia* Kunth pertain to a distinct and divergent lineage whose oldest validly published name is *Rumicastrum* Ulbrich. In 1998, more than half of accepted species were transferred erroneously to a new genus, *Parakeelya* Hershk. However, taxonomists and databases have continued to classify the species in *Calandrinia*, confounding the taxonomy of the latter. Here, 65 Australian species classified in *Calandrinia* are transferred to *Rumicastrum*. This consummates the phylogenetic revision of Montiaceae taxonomy initiated more 30 years ago. Published on-line www.phytologia.org *Published on-line www.phytologia.org Phytologia 102(3): 116-123 (Sept 21, 2020). ISSN 030319430.*

KEY WORDS: *Rumicastrum*, *Calandrinia*, *Parakeelya*, Montiaceae, Australia.

Carolin (1987) published a seminal analysis of classical Portulacaceae (including Montiaceae) that found, among other things, polyphyly of the classical circumscription of the genus *Calandrinia* Kunth, which then included ca.120 accepted species. He determined that the 30–35 accepted Australian species then classified in *Calandrinia* represented a lineage distinct and divergent from other members of *Calandrinia* s. lato. He also determined that the monotypic Australian genus *Rumicastrum* Ulbrich [TYPE: *R. chamaecladum* (Diels) Ulbrich]), originally classified in Chenopodiaceae, pertained to this lineage. He prepared but did not publish a manuscript that recombined all named Australian calandrinias in *Rumicastrum*. Hershkovitz (1993) substantially revised Carolin's (1987) analysis and established the current circumscription of *Calandrinia* (see Hershkovitz, 2019), comprising then only ca. 14 accepted New World species. He likewise confirmed that the Australian calandrinias pertained to a distinct lineage.

Hancock et al. (2018) corroborated Carolin's (1987) interpretation using genomic data that sampled broadly the Australian calandrinias and *Rumicastrum*. However, several earlier molecular studies, 1997–2015 (reviewed in Hershkovitz, 2019) sampling fewer Australian species already had found them to be highly divergent from other Montiaceae lineages. But in the meantime, the taxonomy of the Australian species remained in limbo. In Hershkovitz and Zimmer (1997), I was prevented from recombining an Australian calandrinia in *Rumicastrum* by an editor who maintained that *Rumicastrum* pertained to Chenopodiaceae. For this reason, preliminary to submitting an invited treatment of the Australian calandrinias (Hershkovitz, 2002), I transferred the species to a new genus, *Parakeelya* Hershk. (Hershkovitz, 1998). But in 1999, at the International Botanical Congress in St. Louis, J. G. West (CANB) assured me that I had erred and that *Rumicastrum* indeed was an Australian calandrinia. Yet, since then, Australian botanists have described an additional 24 Australian species in *Calandrinia*, including J. G. West and Chinnock (2013), who did not even mention *Rumicastrum* or *Parakeelya*.

Owing to the results of Hancock et al. (2018), Thiele et al. (2018; including J. G. West) recognized that continued classification of the Australian species in *Calandrinia* was untenable. Surprisingly, they have proposed to conserve the generic name *Parakeelya* over *Rumicastrum*, arguing that the former had become more commonly used than the latter. Thus, they argued, switching to the latter would disrupt established taxonomic usage. The argument is a red herring, because *neither* name has been used commonly; overwhelmingly *Calandrinia* is used. Indeed, *Parakeelya* and/or the existing combinations are listed as synonyms of *Calandrinia* and/or combinations therein in current databases

(COL [Catalog of Life], Hassler, 2020; GBIF, GBIF Secretariat, 2017; POWO [Plants of the World Online], POWO, 2019; and WFO, World Flora Online, without date). Most recently, a new Australian species was described in *Calandrinia* by Obbens (2019; coauthor of Thiele, et al. 2018). Obbens (2019) reported that the new species pertained to the same species group as *Rumicastrum chamaecladum*, but did not explain therein why the generic classifications therefore differed.

Scrutiny reveals something peculiar. Up to 30 years before Hancock et al. (2018), all concerned parties (except one unqualified editor) had agreed that the Australian calandrinias form a distinct lineage of Montiaceae, and that this lineage includes *Rumicastrum*. Inexplicably, therefore, Hancock et al. (2018) described *Calandrinia* as a genus comprising only *Calandrinia* sensu Hershkovitz (1993) plus the Australian calandrinias, and excluding *Rumicastrum*. Conceptually, this was a *new* genus; there *never* had been a proposal to combine just these two particular lineages. And Hancock et al. (2018) obviously knew the circumscription was erroneous before they proposed it, and indeed, their article rejected it.

Both the posture of Thiele et al. (2018) and the *Calandrinia* circumscription of Hancock et al. (2018) are puzzling. The authors clearly prefer *Parakeelya*. So why have they not applied the name they prefer? And, seemingly contradictorily, why do they then cite frequent historical application as the reason for its nomenclatural conservation? Evidently because they realize that *Parakeelya* is not monophyletic without *Rumicastrum*, which still has priority. But no law, nomenclatural or otherwise, imposes phylogeny as a taxonomic criterion. This is the authors' preference. In the meantime, in contradiction to this preference, they have used and continue to use a contrived circumscription of *Calandrinia* they know is *not* monophyletic. Hershkovitz (2019, 2020) surmised that Thiele et al. (2018) have avoided using *Rumicastrum* (except for *R. chamaecladum*) not for scientific, but for aesthetic reasons, and have bended science in the process. The result is that more than 30 years after Carolin's (1987) proposal, the taxonomy of *Calandrinia* at the *global* scale remains unnecessarily muddled.

Here, I complete what Roger Carolin started in his unpublished manuscript, providing below recombinations of all accepted names (per POWO) of Australian calandrinias in *Rumicastrum*. This also corrects my erroneous classification of the species in *Parakeelya* (Hershkovitz, 1998) and also *finally* consummates Carolin's (1987) phylogenetic revision of Montiaceae taxonomy. It is a shame that Carolin or I had not published these recombinations decades ago, in Carolin's case because of his retirement, in mine because of my unjustified deference to my specialist Montiaceae colleagues (Hershkovitz, 2019: 50). To recognize Carolin's contribution, I credit him as the author for all names in *Rumicastrum* included in his manuscript. This, especially, renders them *beautiful* names for these lovely and remarkable plants.

Rumicastrum Ulbrich, Nat. Pflanzenfam., ed. 2 [Engler & Prantl] 16c: 519. 1934. TYPE: *Rumicastrum chamaecladum* (Diels) Ulbrich ≡ *Atriplex chamaeclada* Diels, Repert. Spec. Nov. Regni Veg. 16: 194. (31 Dec.) 1919. [= *Calandrinia* sect. *Apicales* Poelln., Repert. Spec. Nov. Regni Veg. 35: 164. (15 June) 1934. TYPE: non design.; = *Calandrinia* sect. *Basales* Poelln., Repert. Spec. Nov. Regni Veg. 35: 164. (15 June) 1934. TYPE: non design.; = *Calandrinia* sect. *Tuberosae* Poelln., Repert. Spec. Nov. Regni Veg. 35: 165. (15 June) 1934. TYPE: non design.; = *Calandrinia* sect. *Pseudo-dianthoideae* Poelln., Repert. Spec. Nov. Regni Veg. 35: 166. (15 June) 1934. TYPE: non design.; = *Calandrinia* sect. *Uniflorae* Poelln., Repert. Spec. Nov. Regni Veg. 35: 165. (15 June) 1934. TYPE: *C. uniflora* F. Muell., Trans. & Proc. Philos. Inst. Victoria 3: 41. 1859.; = *Parakeelya* Hershk., Phytologia 84(2): 101. (Feb.) 1998. TYPE: *P. ptychosperma* (F. Muell.) Hershk. ≡ *Calandrinia ptychosperma* F. Muell., Fragm. 4(29): 137. (Nov.) 1864. ≡ *Claytonia ptychosperma* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882.

Rumicastrum arenicolum (Syeda) Hershk., comb. nov. Basionym: *Calandrinia arenicola* Syeda, Proc. Linn. Soc. New South Wales 116: 153. 1996. ≡ *Parakeelya arenicola* (Syeda) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum baccatum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia baccata* Obbens, Nuytsia 24: 37. (1 May) 2014.

Rumicastrum balonense (Lindl.) Carolin, comb. nov. Basionym: *Calandrinia balonensis* Lindl. in T. L. Mitchell, J. Exped. Trop. Australia. 148. (16 Feb.–31 Mar.) 1848. ≡ *Claytonia balonnensis* (Lindl.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya balonensis* (Lindl.) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum brevipedatum (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia brevipedata* F. Muell., Fragm. 10(84): 69. (July) 1876. ≡ *Claytonia brevipedata* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya brevipedata* (F. Muell.) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum butcherense (Obbens) Hershk., comb. nov. Basionym: *Calandrinia butcherensis* Obbens, Nuytsia 24: 208. (21 Aug.) 2014. [NOTE: For reasons not specified, IPNI lists *Calandrinia butcherensis* as "nom. inval."; POWO lists it as an "unplaced name." It was published together with *Calandrinia rubrisabulosa* Obbens, which is accepted by both databases, and I am unable to detect the nomenclatural flaw in the publication of *C. butcherensis*.]

Rumicastrum calyptratum (Hook. f.) Carolin, comb. nov. Basionym: *Calandrinia calyptrata* Hook. f. in Hook., Icon. Pl. 3: 296. 1840. ≡ *Claytonia calyptrata* (Hook. f.) F. Muell., Fragm. 3(20): 89. (Sept.) 1862. ≡ *Parakeelya calyptrata* (Hook. f.) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum compositum (Nees) Carolin, comb. nov. Basionym: *Calandrinia polypetala* Fenzl in Endl. et al. var. *composita* Nees in Lehm., Pl. Preiss. 1: 247. (9–11 Feb.) 1845. ≡ *Claytonia composita* (Nees) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya composita* (Nees) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum corrigioloides (F. Muell. ex Benth.) Carolin, comb. nov. Basionym: *Calandrinia corrigioloides* F. Muell. ex Benth., Fl. Austral. 1: 175. (30 May) 1863. ≡ *Claytonia corrigioloides* (F. Muell. ex Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya corrigioloides* (F. Muell. ex Benth.) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum creethae (Tratman ex Morrison) Carolin, comb. nov. Basionym: *Calandrinia creethae* Tratman ex Morrison, J. Bot. 50: 165. (May) 1912. ≡ *Parakeelya creethae* (Tratman ex Morrison) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum crispisepalum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia crispisepala* Obbens, Nuytsia 16(1): 100. (30 Dec.) 2006.

Rumicastrum cygnorum (Diels) Carolin, comb. nov. Basionym: *Calandrinia cygnorum* Diels in Diels & E. Pritz., Bot. Jahrb. Syst. 35(2-3): 199. (6 Dec.) 1904.

Rumicastrum cylindricum (Poelln.) Carolin, comb. nov. Basionym: *Calandrinia cylindrica* Poelln., Repert. Spec. Nov. Regni Veg. 35: 163. (15 June) 1934.

Rumicastrum dielsii (Poelln.) Carolin, comb. nov. Basionym: *Calandrinia dielsii* Poelln., Repert. Spec. Nov. Regni Veg. 35: 162. (15 June) 1934.

Rumicastrum dipetalum (J. M. Black) Carolin, comb. nov. Basionym: *Calandrinia dipetala* J. M. Black, Trans. & Proc. Roy. Soc. S. Austral. 51: 379. 1927.

Rumicastrum dispermum (J. M. Black) Carolin, comb. nov. Basionym: *Calandrinia disperma* J. M. Black, Trans. & Proc. Roy. Soc. South Australia 45: 11, t. 3. 1921. ≡ *Parakeelya disperma* (J. M. Black) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum eremaeum (Ewart) Carolin, comb. nov. Basionym: *Calandrinia eremaea* Ewart. Fl. Victoria 486. (Apr.) 1931 [1930]. ≡ *Calandrinia pusilla* Lindl., in T. L. Mitchell, J. Exped. Trop. Australia. 360. (16 Feb.–31 Mar.) 1848, nom. illegit., non *Calandrinia pusilla* Barnéoud in Gay, Fl. Chil. 2(4): 485. 1847 [1846]. ≡ *Parakeelya eremaea* (Ewart) Hershk., Phytologia 84(2): 101. (Feb.) 1998.

Rumicastrum flavum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia flava* Obbens, Nuytsia 21(1): 2. (24 June) 2011.

Rumicastrum gracile (Benth.) Carolin, comb. nov. Basionym: *Calandrinia gracilis* Benth., Fl. Austral. 1: 173. (30 May) 1863. ≡ *Claytonia gracilis* (Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya gracilis* (Benth.) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum granuliferum (Benth.) Carolin, comb. nov. Basionym: *Calandrinia granulifera* Benth., Fl. Austral. 1:176. (30 May) 1863. ≡ *Claytonia granulifera* (Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya granulifera* (Benth.) Hershk., Phytologia 84(2): 102. (Feb.) 1998. [= *Talinum nanum* Nees in Lehm., Pl. Preiss. 1: 246. (9–11 Feb.) 1845. ≡ *Calandrinia pygmaea* F. Muell. Fragm. 1(7):175. (Sept.) 1859, nom. illegit. ≡ *Calandrinia neesiana* Eichler, ref. ≡ *Parakeelya nana* (Nees) Hershk., Phytologia 84(2): 102. (Feb.) 1998.]

Rumicastrum holtumii (Obbens & L. P. Hancock) Hershk., comb. nov. Basionym: *Calandrinia holtumii* Obbens & L. P. Hancock, Nuytsia 28: 218. (8 June) 2017.

Rumicastrum hortiorum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia hortiorum* Obbens, Nuytsia 22(6): 352. (18 Dec.) 2012.

Rumicastrum kalanniense (Obbens) Hershk., comb. nov. Basionym: *Calandrinia kalanniensis* Obbens, Nuytsia 16(1): 102. (18 Dec.) 2006.

Rumicastrum lefroyense (Obbens) Hershk., comb. nov. Basionym: *Calandrinia lefroyensis* Obbens, Nuytsia 29: 198. (13 July) 2018.

Rumicastrum lehmannii (Endl.) Carolin, comb. nov. Basionym: *Calandrinia lehmannii* Endl. in Lehm., Pl. Preiss. 2(2–3): 235. (2–5 Aug.) 1848. ≡ *Claytonia lehmannii* (Endl.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya lehmannii* (Endl.) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum liniflorum (Fenzl) Carolin, comb. nov. Basionym: *Calandrinia liniflora* Fenzl in Endl. et al., Enum. Pl. 52. (Apr.) 1837. ≡ *Claytonia liniflora* (Fenzl) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya liniflora* (Fenzl) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum maryonii (S. Moore) Carolin, comb. nov. Basionym: *Calandrinia maryonii* S. Moore, J. Linn. Soc., Bot. 45: 164. (7 Dec.) 1920.

Rumicastrum mirabile (Chinnock & J. G. West) Hershk., comb. nov. Basionym: *Calandrinia mirabilis* Chinnock & J. G. West, J. Adelaide Bot. Gard. 26(4): 97. 2013.

Rumicastrum monogynum (Poelln.) Carolin, comb. nov. Basionym: *Calandrinia monogyna* Poelln., Repert. Spec. Nov. Regni Veg. 35: 163. (15 June) 1934.

Rumicastrum monospermum (Syeda ex Obbens) Hershk., comb. nov. Basionym: *Calandrinia monosperma* Syeda ex Obbens, Nuytsia 30: 238. (15 Oct.) 2019.

Rumicastrum morrisae (Goy) Carolin, comb. nov. Basionym: *Calandrinia morrisae* Goy, Proc. Roy. Soc. Queensland 50: 68. (8 June) 1939.

Rumicastrum oblongum (Syeda & Carolin) Hershk., comb. nov. Basionym: *Calandrinia oblonga* Syeda & Carolin, Proc. Linn. Soc. New South Wales 133: 11. 2012 [2011].

Rumicastrum opertum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia operta* Obbens, Nuytsia 22(6): 359. (18 Dec.) 2012.

Rumicastrum orarium (Obbens) Hershk., comb. nov. Basionym: *Calandrinia oraria* Obbens, Nuytsia 24: 41. (1 May) 2014.

Rumicastrum papillatum (Syeda) Carolin, comb. nov. Basionym: *Calandrinia papillata* Syeda, Telopea 2(1): 60. 1980. ≡ *Parakeelya papillata* (Syeda) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum pentavalve (Obbens) Hershk., comb. nov. Basionym: *Calandrinia pentavalvis* Obbens, Nuytsia 21(1): 9. (24 June) 2011.

Rumicastrum pickeringii (A. Gray) Carolin, comb. nov. Basionym: *Calandrinia pickeringii* A. Gray, U.S. Expl. Exped., Phan. 1: 144. 1854. ≡ *Claytonia pickeringii* (A. Gray) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya pickeringii* (A. Gray) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum pleiopetalum (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia pleiopetala* F. Muell, Fragm. 10(84): 10. (July) 1876. ≡ *Claytonia pleiopetala* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya pleiopetala* (F. Muell.) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum polyandrum (Benth.) Carolin, comb. nov. Basionym: *Calandrinia polyandra* Benth., Fl. Austral. 1: 172. (30 May) 1863. ≡ *Talinum polyandrum* Hook., Bot. Mag. 4833. 1855, nom. illegit. [non *Talinum polyandrum* Ruiz & Pav., Syst. Veg. Fl. Peruv. Chil. 115. (late Dec) 1798.] ≡ *Claytonia polyandra* (Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya polyandra* (Benth.) Hershk., Phytologia 84(2): 102. (Feb.) 1998. [= *Calandrinia polyandra* var. *leptophylla* Benth., Fl. Austral. 1: 173. (30 May) 1863.]

Rumicastrum polypetalum (Fenzl) Carolin, comb. nov. Basionym: *Calandrinia polypetala* Fenzl in Endl. et al., Enum. Pl. 51. (Apr.) 1837. ≡ *Claytonia polypetala* (Fenzl) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya polypetala* (Fenzl) Hershk., Phytologia 84(2): 102. (Feb.) 1998.

Rumicastrum poriferum (Syeda) Carolin, comb. nov. Basionym: *Calandrinia porifera* Syeda, Telopea 2(1): 59. 1980. ≡ *Parakeelya porifera* (Syeda) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum primuliflorum (Diels) Carolin, comb. nov. Basionym: *Calandrinia primuliflora* Diels in Diels & E. Pritz., Bot. Jahrb. Syst. 35(2–3): 198. (6 Dec.) 1904. ≡ *Parakeelya primuliflora* (Diels) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum ptychospermum (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia ptychosperma* F. Muell., Fragm. 4(29): 137. (Nov.) 1864. ≡ *Claytonia ptychosperma* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya ptychosperma* (F. Muell.) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum pumilum (Benth.) Carolin, comb. nov. Basionym: *Calandrinia calyptrata* Hook. f. var. *pumila* Benth., Fl. Austral. 1: 175. (30 May) 1863. ≡ *Claytonia pumila* (Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya pumila* (Benth.) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum quadrivalve (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia quadrivalvis* F. Muell., Fragm. 1(7): 176. (Sept.) 1859. ≡ *Claytonia quadrivalvis* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Cosmia quadrivalvis* (F.Muell.) Britten, J. Bot. 38: 77. 1900. ≡ *Parakeelya quadrivalvis* (F. Muell.) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum quartziticum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia quartzitica* Obbens, Nuytsia 29: 194. (13 July) 2018.

Rumicastrum remotum (J. M. Black) Carolin, comb. nov. Basionym: *Calandrinia remota* J. M. Black, Trans. & Proc. Roy. Soc. South Australia 47: 369. 1923. ≡ *Parakeelya remota* (J. M. Black) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum reticulatum (Syeda) Carolin, comb. nov. Basionym: *Calandrinia reticulata* Syeda, Telopea 2(1): 60. 1980. ≡ *Parakeelya reticulata* (Syeda) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum rubrisabulosum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia rubrisabulosa* Obbens, Nuytsia 24: 210. (21 Aug.) 2014.

Rumicastrum schistorhizum (Morrison) Carolin, comb. nov. Basionym: *Calandrinia schistorhiza* Morrison, J. Bot. 50: 164. (May) 1912. ≡ *Parakeelya schistorhiza* (Morrison) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum sculptum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia sculpta* Obbens & J. G. West, Nuytsia 21(1): 12. (24 June) 2011.

Rumicastrum spergularinum (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia spergularina* F. Muell, Fragm. 1(7): 175. (Sept.) 1859. ≡ *Claytonia spergularina* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya spergularina* (F. Muell.) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum sphaerophyllum (J. M. Black) Carolin, comb. nov. Basionym: *Calandrinia sphaerophylla* J. M. Black, Trans. & Proc. Roy. Soc. South Australia 51: 378. 1927. ≡ *Parakeelya sphaerophylla* (J. M. Black) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum stagnense (J. M. Black) Carolin, comb. nov. Basionym: *Calandrinia stagnensis* J. M. Black, Trans. & Proc. Roy. Soc. South Australia 51: 379. 1927. ≡ *Parakeelya stagnensis* (J. M. Black) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum stenogynum (Domin) Carolin, comb. nov. Basionym: *Calandrinia stenogyna* Domin, Biblioth. Bot. 22(89): 97. (Jan.) 1926 [1925]. ≡ *Parakeelya stenogyna* (Domin) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum strophiolatum (F. Muell.) Carolin, comb. nov. Basionym: ≡ *Claytonia strophiolata* F. Muell., Fragm. 11(91): 82. (Feb.) 1880. ≡ *Calandrinia strophiolata* (F. Muell.) Poelln., Feddes Repert. Spec. Nov. Regni Veg. 35: 173. 1934. ≡ *Parakeelya strophiolata* (F. Muell.) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum tepperianum (W. Fitzg.) Carolin, comb. nov. Basionym: *Calandrinia tepperiana* W. Fitzg., J. Proc. Roy. Soc. Western Australia 3: 141. 1918 [1916–1917].

Rumicastrum tholiforme (Obbens) Hershk., comb. nov. Basionym: *Calandrinia tholiformis* Obbens, Nuytsia 21(1): 6. (24 June) 2011.

Rumicastrum translucens (Obbens) Hershk., comb. nov. Basionym: *Calandrinia translucens* Obbens, Nuytsia 16(1): 104. (20 Dec.) 2006.

Rumicastrum tumidum (Syeda) Hershk., comb. nov. Basionym: *Calandrinia tumida* Syeda, Proc. Linn. Soc. New South Wales 116: 156. 1996. ≡ *Parakeelya tumida* (Syeda) Hershk., Phytologia 84(2): 103. (Feb.) 1998.

Rumicastrum umbelliforme (Obbens) Hershk., comb. nov. Basionym: *Calandrinia umbelliformis* Obbens, Nuytsia 22(6): 356. (18 Dec.) 2012.

Rumicastrum uncinellum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia uncinella* Obbens, Nuytsia 30: 242. (15 0ct.) 2019.

Rumicastrum uniflorum (F. Muell.) Carolin, comb. nov. Basionym: *Calandrinia uniflora* F. Muell., Trans. & Proc. Philos. Inst. Victoria 3: 41. 1859. ≡ *Claytonia uniflora* (F. Muell.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya uniflora* (F. Muell.) Hershk., Phytologia 84(2): 104. (Feb.) 1998.

Rumicastrum vernicosum (Obbens) Hershk., comb. nov. Basionym: *Calandrinia vernicosa* Obbens, Nuytsia 21(1): 15. (24 June) 2011.

Rumicastrum volubile (Benth.) Carolin, comb. nov. Basionym: *Calandrinia volubilis* Benth., Fl. Austral. 1: 174. (30 May) 1863. ≡ *Claytonia volubilis* (Benth.) F. Muell., Syst. Census Austral. Pl. 27. 1882. ≡ *Parakeelya volubilis* (Benth.) Hershk., Phytologia 84(2): 104. (Feb.) 1998. [= *Calandrinia volubilis var. parvula* J. M. Black, Trans. & Proc. Roy. Soc. South Australia 49: 272. 1925.]

Rumicastrum wilsonii (Obbens) Hershk., comb. nov. Basionym: *Calandrinia wilsonii* Obbens, Nuytsia 29: 201. (13 July) 2018.

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The leaf volatile terpenoids of *Pinus heldreichii* Christ from Bulgaria and comparisons with Greece and Montenegro-Serbia oils, and *P. leucodermis* oil, Italy.

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ABSTRACT

A detailed, complete analysis of volatile leaf oils of *Pinus heldreichii* from Bulgaria found the oil very high in limonene (46.5%), germacrene D (15.4%) and α-pinene (14.3%). The oil is similar to literature reports from Greece, Montenegro-Serbia and Bulgaria. Comparison with *P. h.* var. *leucodermis* (southern Italy) revealed many differences in concentrations of terpenoids. The differentiation in the oils of *Pinus heldreichii* and of *P. heldreichii* var. *leucodermis* (southern Italy) supports the recognition of var. *leucodermis* as a distinct taxon, but additional research is needed. While sampling, one tree (14734) was found that appeared to be a hybrid between *Pinus heldreichii* and *P. mugo*. Analysis of its volatile leaf oils revealed that 14734 has 10 compounds that are intermediate in concentration between *Pinus heldreichii* and *P. mugo*, 8 compounds are transgressive (shaded red in Table 2, i.e., larger than in either parent), 2 compounds are about the same concentration as in *P. heldreichii,* and 3 compounds are about the same concentration as in *P. mugo*. Taken together, these data strongly support that plant 14734 is a hybrid between *P. heldreichii var. heldreichii* and *P. mugo*.

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KEY WORDS: *Pinus heldreichii, P. mugo* hybrids, Bulgaria, volatile leaf oil, terpenes, composition, *P. leucodermis.*

Pinus heldreichii Christ was named after its discovered, von Heldreich in the mountains of northern Greece (Gymnosperm Database, 2019). It is rare and endangered in its range in the Balkans. A variety, *P. heldreichii* var. *leucodermis* (Antoine) Markgraf ex Fitschen, grows in the Calabrian region of southern Italy, the Balkans and Greece. It is often treated as a distinct species (*P. leucodermis* Antoine) in Italy (Pennacchini and Bonin, 1975).

There has been considerable research on the volatile leaf oils of *Pinus heldreichii* (see Mitic et al., 2017; Nikolic et al. 2011, for recent literature reviews). Petrakis, et al. (2001) examined the volatile oil of *P. heldreichii* growing on Mt. Katara, central Greece and reported the oil was dominated by limonene (34.3%), α-pinene (16.7%), germacrene D (12.8%), and (E)-caryophyllene (8.4%). This is similar to the report by Mitic et al. (2017) who analyzed plants from Montenegro and Serbia and noted the oils were dominated by limonene (25.8%), α-pinene (16.0%), germacrene D (15.3%), and (E)-caryophyllene (10.2%). Ioannou et al. (2014) reported similar composition from Mt. Pindos, Metsovo, Greece.

Nikolic et al. (2011) examined variation in the leaf oils of *P. heldreichii* in Montenegro and Serbia and reported the most distinguishing terpenes were germacrene D (13.5%), myrcene (2.2%), α-humulene (2.1%), α-muurolene (1.7%), α-muurolene (1.2%), γ-cadinene (1.0%), α-cadinene (0.5), and β-bourbonene (0.3%). They did not publish a complete analysis of *P. heldreichii* volatile leaf oil.

Naydenov et al. (2005) reported on chloroplast microsatellites and terpene analysis from *P. heldreichii* from Bulgaria. Thet found the volatile leaf oil was dominated by limonene (36.9 - 48.2%) and α-pinene (16.9 - 18.6%) and no significant correlation between cp microsatellite (cpSSR) and terpene variation patterns. They reported on the composition of only 10 terpenoids: α-pinene (16.9-18.6%), camphene (1.86 - 2.23%), β-pinene (5.07 - 6.49%), δ-3-carene (3.20 - 4.96%), limonene, 36.9 - 48.2%), terpinolene (0.85 - 1.01%), β-farnesene (4.73 - 7.64%), β-selinene (0.75 - 1.33%), γ-muurolene (14.85 - 22.87%), γ-cadinene (1.36 - 2.32%).

Searches of the literature revealed only one paper (Bonesi et al., 2010) reporting on the composition of *P. leucodermis* from southern Italy).

The purpose of this report, a continuation of research on leaf volatile oils of *Pinus* in Bulgaria (Adams and Tashev, 2019a,b), is to present a complete analysis of the volatile leaf of *Pinus heldreichii* from Bulgaria and compare its composition with reports from Montenegro-Serbia, and with the oil of *P. heldreichii* var. *leucodermis* (southern Italy). In the midst of this study, we found a tree with oil that was intermediate in composition between *P. heldreichii* and *P. mugo,* so the composition of this putative hybrid along with two nearby *P. mugo* tree's oils is also presented.

MATERIALS AND METHODS

Leaf samples of *P. heldreichii* collected:
BULGARIA: Pirin Mountain (North), National Park "Pirin". Between the huts "Banderitza" and "Vihren", in the valley of Banderishka river, together with *Pinus peuce, P. mugo, Juniperus communis*. 41°45'57.9" N, 23°25'26.1" E., 1815 m,18 May 2015. Coll. *Alexander & Nikolay Tashev* 2015 Sp.1-3 *PH1-PH5, Lab Acc. Robert P. Adams 14732-14736.* Population of *P. heldreichii, P. mugo* and a putative hybrid.
BULGARIA: North Pirin Mt., Bahderishka, glade location. 41° 45' 46" N to 41° 45' 57" N; 23° 25' 6" E, 23° 25' 22" E, 1807-1863 m. 12 Oct 2019, Coll. Alexander et. Nikolay Tashev Ph1-Ph10, Lab Acc. *Robert P Adams 15832-15841*. Population of only *P. heldreichii,* used to obtain a composite of oil *15832-15841* (10) to represent *P. heldreichii* oil.
Voucher specimens are deposited in the herbarium, University of Forestry, Dept. of Dendrology, 10, Kliment Ochridsky Blvd., 1797 Sofia, Bulgaria

Gently dried leaves (100g, 40 - 45°C) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details, Out of print, free pdf: www.juniperus.org). Identifications were made by library searches of the Adams volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Limonene, sylvestrene and β-phellandrene co-elute as a single peak on DB-5, but their amounts can be quantitated by Single Ion Chromatography (SIC, Adams and Tashev 2019b). Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

The composition of the volatile leaf essentials oils of *P. heldreichii* from Bulgaria are shown in Table 1 in comparison with oils from Bulgaria (Naydenov et al. 2005), Greece (Petrakis et al. 2001), and Montenegro-Serbia (Metic, et al. 2017) and *P. heldreichii* var. *leucodermis,* Italy (Bonesi et al., 2010).

The oil composition of *P. heldreichii* var. *heldreichii* is very similar to the oil compositions reported from Bulgaria, Greece and Montenegro-Serbia (Table 1). It should be noted that the report of 18.4% γ-muurolene (Table 1, Naydenov, et al. 2005), is likely germacrene D, because the other reports (Table 1) list γ-muurolene as varying from 0.0 to 0.9%, and germacrene D ranging from 12.8% to 15.2% for *P. heldreichii.* The mass spectra of γ-muurolene and germacrene D are very similar, as are their retention times on DB5 (1478 and 1480) (Adams, 2007). It is easy to mis-identify these compounds.

It is of interest that the oil of *P. h.* var. *leucodermis* (southern Italy, Bonesi et al. 2010) are quite different from that of *P. heldreichii* from any location in numerous constituents (green, Table 1). The var. *leucodermis* (= *P. leucodermis* in many flora treatments) oil sample report (Bonesi et al. 2010)from southern Italy differed in many components from the oils of *P. heldreichii* (cpd, *leucodermis, heldreichii* range): α-pinene (24.2, 9.6-16.0%), limonene (7.8, 25.8-46.5%), (E)-β-ocimene (3.7, 0-0.2%), terpinolene (5.9, 0-0.6%), terpinen-4-ol (0.8, 0-0.2%), α-terpineol (1.7, 0-0.2%), linalool acetate (3.6, 0), bornyl acetate (2.7,0 - 0.7%), myrtenyl acetate (0.6%, 0%), α-cubebene (7.6; 0%), germacrene D (0.7, 12.8 - 15.4%), ethyl dodecanoate (0.3; 0-trace%), tetradecanal (0.5; 0-trace%) and manool oxide (0.6; 0-trace%). There are clearly differences in the volatile leaf oils between *P. heldreichii* var. *heldreichii,* var. *leucodermis* based on this study *and* Bonesi et al. 2010. Additional samples are needed of var. *leucodermis* to verify these differences. According to Farjon (1984) and Richardson and Rundel (1998), *P. h.* var. *leucodermis* grows in southern Italy, the Balkans and Greece, on sunny, dry slopes. Janković (1986) states that var. *leucodermis* grows in pure stands only on steep, dry rocky southern slopes. Boscherini et al. (1994) reported no variation in cpDNA amplifications and restriction patterns among all *P. leucodermis* individuals sampled from seven populations (two in Greece and five in Italy). This is remarkable, and indicates that whatever the taxonomic status of *P. leucodermis*, the same taxon grows in Italy and Greece. Unfortunately, they did not include any samples of *P. heldreichii* and so, were not able to determine if these taxa differ in the cpDNA characters.

A detailed examination of leaf oil variation among plants in a Bulgaria population revealed a tree with oil that was intermediate in composition between *P. heldreichii* and *P. mugo,* so the composition of this putative hybrid, along with two nearby *P. mugo* tree's oils were analyzed (analyses of other *P. mugo* trees are reported in Adams and Tashev (2019a). Although morphology is usually intermediate in hybrids, in two recent studies on the inheritance of terpenoids in *Cryptomeria japonica* (Adams and Tsumura, 2012) and *Pseudotsuga menziesii* (Adams and Stoehr, 2013) artificial hybrids revealed that many compounds are transgressive in the hybrids, (i.e., greater (or lower) concentration than found in either parent), the same concentration as one of the parents, or intermediate in concentration between the parents' values.

Examination of Table 2 reveals: 10 compounds are intermediate (shaded aqua): tert-butyl-benzene, sabinene, α-phellandrene, δ-3-carene, limonene, (E)-β-ocimene, terpinolene, m-cymen-8-ol, p-cymen-8-ol and bornyl acetate. Eight compounds are transgressive (shaded red in Table 2, i.e., larger than either parent in this case): myrcene, β-phellandrene, α-terpinyl acetate, β-elemene, bicyclogermacrene, γ-cadinene, δ-cadinene, and (E)-nerolidol. Two compounds are about the same concentration as in *P. heldreichii* (trace amount): α-thujene and cis-carveol, and 3 compounds are about the same concentration as in *P. mugo*: p-cymene, germacrene D, and α-muurolene (Table 2). Taken together, these data strongly support that plant 14734 is a hybrid between *P. heldreichii var. heldreichii* and *P. mugo*. It is very likely that other hybrids are in the area.

The differentiation reported in the oil of *P. h.* var. *leucodermis* (southern Italy) supports the recognition of this taxon as a distinct species, but additional research is needed to determine if its oil maintains its compositional profile throughout its range from Italy to Greece.

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Table 1. The leaf oil constituents of *Pinus heldreichii* from Bulgaria compared with other analyses on the volatile leaf oils. Compounds highlighted in yellow/green separate var. *heldreichii* and var. *leucodermis*, those with aqua highlight are unusual amounts. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported. KI is the Kovat's Index using a linear calculation on DB-5 column.

KI	compound	Bulgaria comp oil 10 trees 15868	Bulgaria ex Naydenov[1]	Greece Mt. Pindos Ioannou[2]	Greece ex Petrakis[3]	Montenegro Serbia ex Metic[4]	*P. h.* var. *leucodermis* Italy Bonesi[4]
921	tricyclene	0.2	-	0.3	-	-	-
924	α-thujene	t	-	-	0.3	0.2	-
932	**α-pinene**	**14.3**	**17.4**	**0.1**	**13.8**	**16.0**	**24.2**
946	camphene	0.7	2.0	0.8	1.5	0.9	0.4
948	benzaldehyde	t	-	-	-	-	-
967	(tert-butyl benzene)	t	-	-	-	-	-
969	sabinene	t	-	0.6	0.8	0.1	0.1
974	**β-pinene**	**3.8**	**5.8**	**3.6**	**4.2**	**5.2**	**8.4**
988	myrcene	2.4	-	2.2	2.5	2.2	-
1002	α-phellandrene	t	-	-	-	0.0	-
1008	**δ-3-carene**	t	**4.3**	18.6	**2.8**	**2.1**	**0.2**
1014	α-terpinene	t	-	0.2	-	-	1.5
1020	p-cymene	t	-	t	-	-	0.2
1023	silvestrene	t	-	-	-	-	-
1024	limonene	46.5	42.0	23.7	34.3	25.8	7.8
1025	β-phellandrene	-	0.9	-	t	0.0	t
1044	(E)-β-ocimene	t	-	-	0.1	0.2	3.7
1054	γ-terpinene	t	-	0.3	0.1	-	1.0
1065	cis-sabinene hydrate	t	-	-	-	-	-
1082	m-cymenene	t	-	-	-	-	-
1086	terpinolene	0.2	-	2.1	0.6	0.5	5.9
1095	linalool	-	-	-	t	-	0.5
1118	trans-p-mentha-2,8-dien-1-ol	t	-	-	-	-	-
1118	cis-p-menth-2-en-1-ol	t	-	-	-	-	-
1122	α-campholenal	t	-	-	-	-	0.1
1126	cyclohexene <4-acetyl-1-me-1-	t	-	-	-	-	-
1135	trans-pinocarveol	t	-	-	-	-	0.2
1136	trans-sabinol	t	-	-	-	-	-
1133	cis-p-mentha-2,8-dien-1-ol	t	-	-	-	-	-
1141	camphor	t	-	-	-	-	-
1145	camphene hydrate	t	-	-	-	-	-
1160	pinocamphone	t	-	-	-	-	-
1165	borneol	t	-	-	t	-	-
1174	terpinen-4-ol	t	-	0.1	0.1	-	0.8
1176	m-cymen-8-ol	-	-	-	-	-	-
1179	p-cymen-8-ol	-	-	-	-	-	-
1186	α-terpineol	0.2	-	t	0.2	-	1.7
1195	myrtanol	t	-	-	-	-	-
1215	trans-carveol	t	-	-	-	-	-
1226	cis-carveol	t	-	-	-	-	-
1232	thymol, methyl ether	-	-	-	-	-	0.2
1239	carvone	t	-	-	-	-	-
1253	trans-sabinene hydrate acetate	-	-	-	-	-	-
1254	linalool acetate	-	-	-	-	-	3.6
1284	bornyl acetate	0.7	-	0.3	-	0.1	2.7
1293	2-undecanone	t	-	-	-	-	-
1309	p-vinyl guaiacol	t	-	-	-	-	-
1315	(2E,4E)-decadienal	t	-	-	-	-	-
1324	myrtenyl acetate	-	-	-	-	-	0.6
1345	α-terpinyl acetate	0.3	-	-	0.4	0.5	-
1348	α-cubebene	-	-	-	-	-	7.6
1350	citronellyl acetate	-	-	-	0.2	-	-
1374	α-copaene	t	-	t	-	-	0.4
1379	geranyl acetate	t	-	-	-	-	0.4
1387	β-bourbonene	t	-	t	-	-	0.5
1389	β-elemene	t	-	t	-	-	-

KI	compound	Bulgaria comp oil 10 trees 15868	Bulgaria ex Naydenov[1]	Greece Mt. Pindos Ioannou[2]	Greece ex Petrakis[2]	Montenegro Serbia ex Metic[3]	*P. h.* var. *leucodermis* Italy Bonesi[4]
1403	methyl eugenol	-	-	-	-	-	0.3
1400	β-longipinene	0.5	-	-	-	-	-
1409	α-gurjunene	-	t	-	0.2	-	-
1417	**(E)-caryophyllene**	**5.0**	**-**	**8.6**	**8.4**	**10.2**	**4.5**
1430	β-copaene	t	-	0.1	-	-	-
1431	β-gurjunene	-	-	-	-	1.1	-
1439	aromadendrene	t	-	t	-	0.7	-
1454	α-humulene	0.7	-	1.5	1.0	0.4	1.0
1454	**(E)-β-farnesene**	-	**6.1**	-	**-**	**-**	**0.9**
1465	(E)-ethyl cinnamate	t	-	-	-	-	-
1478	γ-muurolene	t	18.4(GRMD?	0.1	-	0.9	0.2
1480	**germacrene D**	**15.4**	**(18.4)?**	**21.3**	**12.8**	**15.2**	**0.7**
1484	aristolene	-	-	-	6.0	-	-
1489	β-selinene	-	1.1	-	-	-	-
1500	bicyclogermacrene	-	-	-	-	-	-
1500	α-muurolene	0.2	-	0.2	0.3	1.3	-
1508	germacrene A	-	-	-	-	-	-
1513	γ-cadinene	0.2	1.8	0.2	-	0.8	1.0
1522	δ-cadinene	0.4	-	0.6	0.6	1.4	1.8
1537	α-cadinene	t	-	t	-	-	t
1561	(E)-nerolidol	t	-	-	-	-	t
1565	dodecanoic acid	0.4	-	-	-	-	-
1574	germacrene-D-4-ol	t	-	t	-	0.3	t
1583	caryophyllene oxide	t	-	0.2	-	-	t
1594	ethyl dodecanoate (ethyl laurate	t	-	-	-	-	0.3
1608	humulene epoxide II	t	-	-	-	-	-
1611	tetradecanal	-	-	-	-	t	0.5
1638	epi-α-cadinol	t	-	0.1	-	-	-
1640	epi-α-muurolol	t	-	t	-	-	-
1644	α-muurolol	t	-	t	-	-	-
1652	α-cadinol	0.2	-	0.3	-	-	-
1685	germacra-4(15),5,10)14)-trien-1-al	-	-	-	-	-	-
1710	pentadecanal	-	-	-	-	-	-
1713	(2E,6Z)-farnesal	t	-	-	-	-	-
1722	(2Z,6E)-farnesal	1.0	-	-	-	-	-
1740	(2E,6E)-farnesal	t	-	-	-	-	-
1759	benzyl benzoate	-	-	-	-	-	-
1874	hexadecanol	-	-	-	-	-	-
1892	(7Z,10Z,13Z-hexadecatrienal)	t	-	-	-	-	-
1937	cembrene	t	-	t	-	-	-
1943	iso-cembrene	t	-	-	-	-	-
1959	hexadecanoic acid	0.6	-	-	-	-	-
1987	manool oxide	t	-	-	-	-	0.6
2048	thunbergol (isocembrol)	0.2	-	-	-	-	-
2055	abietatriene	t	-	-	-	-	-
2056	manool	0.2	-	-	-	-	-
2087	abietadiene	t	-	-	-	-	-
2116	phytol isomer	0.9	-	-	-	-	-
2220	isopimaral	t	-	-	-	-	-
2243	palustral (8,13-abietadien-18-al	0.2	-	-	-	-	-
2274	dehydro abietal	t	-	-	-	-	-
2310	isopimarol	t	-	-	-	1.3	-
2314	trans-totarol	t	-	-	-	-	-
2381	octadecanoic acid, butyl ester	t	-	-	-	-	-
2420	abietinol acetate (4-epi-dehydro	t	-	-	-	-	-

[1]Naydenov et al. 2005; [2]Ioannou et al. 2014, [3]Petrakis et al. 2001; [4]Mitic et al. 2017; [5]Bonesi et al. 2010.

Table 2. Putative hybridization between *P. heldreichii* and *P. mugo* in a population in Bulgaria. Compounds in yellow are typical of *P. heldreichii*; those in green - *P. mugo*; Compounds in aqua - intermediate; red - transgressive (larger than either parent). Compounds that were always less than 0.5 are not included to simplify the table.

		P. heldreichii			mugo x heldriechii	*P. mugo*		
KI	compound	tree *14732*	tree *14733*	comp oil *15868*(10)	tree *14734*	tree *14735*	tree *14736*	comp oil *15773*(5)
921	tricyclene	0.1	0.2	0.2	0.4	0.5	0.2	0.2
924	α-thujene	t	t	t	t	0.9	0.7	0.3
932	α-pinene	5.0	9.6	14.3	9.4	10.8	7.5	11.0
946	camphene	0.3	0.9	0.7	1.7	1.9	0.7	1.2
967	(tert-butyl benzene)	t	t	t	0.2	0.5	1.1	0.5
969	sabinene	t	0.1	t	0.6	1.4	1.1	1.2
974	β-pinene	1.4	2.3	3.8	3.8	3.3	5.9	2.7
988	myrcene	1.3	1.9	1.9	3.8	2.6	2.0	3.1
1002	α-phellandrene	t	t	t	0.9	0.6	0.4	0.5
1008	δ-3-carene	t	t	t	10.6	27.6	23.1	24.6
1020	p-cymene	t	t	t	0.6	0.7	0.8	0.2
1024	limonene	44.9	54.2	46.5	18.0	t	-	t
1025	β-phellandrene	-	-	-	17.8	14.4	13.1	16.7
1044	(E)-β-ocimene	t	0.1	t	0.5	0.9	0.8	0.6
1054	γ-terpinene	t	t	t	0.5	0.4	0.4	0.4
1086	terpinolene	0.2	0.2	0.2	2.3	3.4	2.9	3.9
1165	borneol	0.7	1.4	t	0.4	0.7	0.5	0.2
1174	terpinen-4-ol	0.2	0.2	t	0.3	0.5	0.6	0.4
1176	m-cymen-8-ol	t	t	-	0.1	0.3	0.7	0.2
1179	p-cymen-8-ol	t	t	-	0.2	0.5	0.7	0.3
1186	α-terpineol	0.7	1.1	0.2	0.5	0.1	0.2	t
1215	trans-carveol	0.3	0.6	t	t	0.2	0.3	t
1226	cis-carveol	0.1	0.3	t	t	0.6	1.2	-
1284	bornyl acetate	0.7	0.3	0.7	2.0	5.5	1.7	4.2
1345	α-terpinyl acetate	0.3	0.4	0.3	1.8	1.4	1.4	1.8
1389	β-elemene	t	t	t	0.6	t	0.2	0.2
1417	(E)-caryophyllene	3.5	4.7	5.0	4.0	5.2	4.9	5.3
1430	β-copaene	0.1	0.1	t	t	t	t	t
1454	α-humulene	0.6	0.8	0.7	0.6	0.9	0.8	0.8
1480	germacrene D	16.7	6.7	15.5	4.7	3.5	4.7	1.7
1500	bicyclogermacrene	0.2	0.1	-	1.8	t	t	1.1
1500	α-muurolene	0.2	0.2	0.2	0.6	0.4	1.2	0.3
1513	γ-cadinene	0.3	0.2	0.2	0.9	t	0.6	0.6
1522	δ-cadinene	0.8	0.3	0.4	1.4	0.2	0.9	1.2
1561	(E)-nerolidol	t	t	t	1.4	0.3	t	0.1
1574	germacrene-D-4-ol	0.2	1.9	t	0.2	0.9	1.8	2.3
1583	caryophyllene oxide	0.2	0.1	t	t	t	1.2	0.4
1652	α-cadinol	0.5	0.5	0.2	0.6	0.2	0.6	1.1
1710	pentadecanal	1.3	0.6	-	0.2	0.2	0.3	0.2
1722	(2Z,6E)-farnesol	0.5	0.2	1.0	t	t	t	t
1874	hexadecanol	2.4	0.3	-	t	0.2	t	t
1959	hexadecanoic acid	1.2	0.2	0.6	0.2	0.2	0.2	-
2116	phytol isomer	0.2	t	0.9	-	t	0.2	-
2243	palustral (8,13-abietadien-18-al	0.3	t	0.2	t	0.2	1.7	1.0

Hybridization between serrate leaf *Juniperus monosperma* and smooth leaf *J. scopulorum* in the Guadalupe Mountains, NM, USA: evidence from DNA sequencing and leaf essential oils

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ABSTRACT

Two unusual *J. scopulorum* trees were discovered in the Guadalupe Mtns., NM and analyses of petN-psbM (cpDNA) confirmed that had chloroplasts (cp) of *J. monosperma*. nrDNA (ITS)sequencing revealed 25 SNPs between *J. monosperma* and *J. scopulorum.* 18 SNPS were analyzed and all SNPs were heterozygous in the 2 unusual plants, implying they are hybrids. In addition, DNA from a 2010 herbarium voucher at UTEP was successfully extracted and sequenced. It contained *J. monosperma* cp and all 18 ITS SNPs were heterozygous, showing it was also a hybrid. Addition analyses of the leaf volatile oils of *J. monosperma, J. scopulorum* plus the 2 putative hybrid trees, confirmed they are hybrids between *J. monosperma* and *J. scopulorum* in the Guadalupe Mtns., NM. Published on-line www.phytologia.org *Published on-line www.phytologia.org Phytologia 102(3):131-142 (Sept 21, 2020). ISSN 030319430.*
KEY WORDS: *Juniperus scopulorum, J. monosperma, nrDNA, petN-psbM,* hybridization, leaf essential oils, Guadalupe Mountains, New Mexico.

Juniperus, in North America, has been the subject of numerous studies on hybridization using morphological data (Fassett, 1944, 1945a, 1945b, Hall 1952; Van Haverbeke 1968; Schurtz 1968).Later studies involved the use of chemical data (Flake et al. 1978; Adams 1983,Palma-Otal et al. 1983; Adams and Kistler 1991, Adams 2013a,b). Recently, DNA sequence data has been used in the study of hybridization between *J. occidentalis* and *J. osteosperma* (Terry et al. 2000; Terry 2010); *J. maritima* and *J. scopulorum* (Adams 2015a, b); *J. scopulorum* and *J. blancoi* (Adams et al. 2020); *J. arizonica* and *J. coahuilensis* (Adams 2017).

The recent study on hybridization and introgression between *J. scopulorum*, in the United States and *J. blancoi* in Mexico utilized nrDNA (ITS region) and cp DNA (petN-psbM, trnS-trnG) sequences for several populations of both species (Adams et al. 2020). Analysis of *J. scopulorum* in the Guadalupe Mountains, NM, found typical *J. scopulorum* DNA, except for two *J. scopulorum* trees with nrDNA intron sites that supported introgression from *J. blancoi* in Mexico (Adams et al. 2020). Additional sampling discovered two other *J. scopulorum* trees that seemed unusual and upon DNA analyses, they appear to be of hybrid origin with nearby *J. monosperma* trees. Because *J. scopulorum* is in the entire leaf margined clade and *J. monosperma* is a member of the serrate leaf junipers clade (Adams 2014), the taxa are, phylogenetically, somewhat remote. Thus, hybridization between these species would see less likely than between, for example, two serrate junipers (*J. arizonica*, *J. coahuilensis*, Adams 2017) or two entire leaf junipers (*J. blancoi*, *J. scopulorum*, Adams et al. 2020).

The purpose of this report is to present both DNA data (nrDNA, cpDNA sequences) and analysis of the leaf essential oils of *J. monosperma and J. scopulorum* to investigate the possible hybrids between *J. monosperma* and *J. scopulorum* in the Guadalupe Mtns., NM, USA.

MATERIAL AND METHODS

Plant material

***J. scopulorum*, Guadalupe Mtns., NM**

Lab Acc. *Robert P. Adams 15602*, ex *Richard Worthington 28617,* UTEP Herbarium accession 58749, Devil's Den Spring, Guadalupe Mtns. 32° 02' 3.12" N, 104° 16' 0.12" W. 2103m(6000ft), 5 Sept. 1999, Eddy County, NM

Lab Acc. *Robert P. Adams 15603*, ex *Richard Worthington 28673*, UTEP Herbarium accession 58750 North Fork, Big Canyon, Guadalupe Mtns. 32° 02' 3.12" N, 104° 45' 0.12" W. 1828m (6000ft), 6 Sept. 1999, Eddy County, NM**.**

Lab acc. *Adams 15783,* *ex George M. Ferguson4624,* with J. Ferguson, Riparian woodland. limestone. male tree, 2 trunks each 30 cm dbh, 9 m tall; bark longitudinally plated, pollen cones forming. Associated species: *Pinus ponderosa var. brachyptera, Pinus edulis, Juniperus deppeana, Quercus muehlenbergii, Quercus grisea, Acer grandidentatum, Berberis haematocarpa, Arbutus xalapensis var. texana, Dasylirion leiophyllum, Agave parryi*. Lincoln National Forest, Guadalupe Mountains, Dark Canyon, S of Klondike Gap near confluence Hooper Canyon, 0.2 mi (by FR 307) E jct County Road 412 (FR 69), just inside USFS boundary. TRS: T25S R21E sec 26 SE1/4, 32° 6' 0" N, 104° 46' 15.6" W.1920m (6300 ft.),3 November 2019, Eddy County, NM

Lab Acc. *Robert P. Adams 15799*, ex *George M. Ferguson 4649*

male tree, 41 cm dbh, 8.5 m tall; bark rough longitudinally plated, dark red beneath, pollen cones just beginning to form, Lincoln National Forest, Guadalupe Mtns., upper Devil's Den canyon, on limestone, Pinyon-oak-juniper woodland w *J. deppeana* and *Pinus* sp., 32° 02' 18.96' N, 104° 48' 5.04" W, 2170m (7120 ft), 19 Jan 2020.Eddy County, NM

***J. monosperma*, Guadalupe Mtns., NM**

Lab Acc. Robert P. Adams 15781ex George M. Ferguson4616 with J. Ferguson, Riparian woodland. limestone. female tree, multiple trunks ca. 20 cm dbh, 4 m tall, bark longitudinally furrowed, cones dark blue with light bloom, 1-seeded. Associated species: *Pinus ponderosa, Pinus edulis, Juniperus deppeana, Quercus muehlenbergii, Quercus grisea, Dasylirion leiophyllum, Agave parryi,* Lincoln National Forest, Guadalupe Mountains, Dark Canyon, 0.4 mi N confluence Goat Canyon on Cougar Road 412 (FR 69). TRS: T25S R22E sec 17 SE1/4, 32° 7' 57"N, 104° 43' 9.84" W. 1768m (5820 ft.), 20 Oct 2019, **Eddy County, NM.**

Lab Acc. *Robert P. Adams 15805, 15806*exColl. *George M. Ferguson 4660, 4661*

female tree, multiple trunks < 10 cm dbh each, 2.5 m tall, bark longitudinally furrowed, cones dark blue with light bloom, 1-seeded (rarely 2), Lincoln National Forest, Guadalupe Mtns., 0.6 mi (by NM 137) W jct FR 540 Guadalupe Ridge Road, at milepost 14.5, on limestone, Pinyon-oak-juniper woodland w *J. deppeana* and *Pinus* sp.32° 9' 43.56" N, 104° 47' 6.36" W, 1868m (6130 ft), 19 Jan 2020.Eddy County, NM

Lab Acc. *Robert P. Adams 15807,* *George M. Ferguson 4662*

male tree, multiple trunks, 15 cm dbh each, 3.5 m tall, bark longitudinally furrowed, pollen cones formed not shedding pollen yet, Lincoln National Forest, Guadalupe Mtns., 1.5 mi (by NM 137) E jct FR 540 Guadalupe Ridge Road, at milepost 16.5, on limestone, Pinyon-oak-juniper woodland w *J. deppeana* and *Pinus* sp. 32° 11' 9.6" N, 104° 46' 6.6" W, 1797m (5895 ft), 19 Jan 2020. County Eddy, NM,

***J. scopulorum* x *J. monosperma*, Guadalupe Mtns., NM**

Lab Acc. *Robert P. Adams 15601*, Coll. Richard Worthington 36160, UTEP Herbarium accession 80150, scale lvs with few very small teeth. otherwise foliage as *scopulorum*. Devil's Den Canyon,

Guadalupe Mtns. 32° 02' 15" N, 104° 47' 54.24" W. ca 2164m (7100ft), 18 July 2010, Eddy County, NM
Lab Acc. Robert P. Adams 15787, ex George M. Ferguson4628 with J. Ferguson
scale lvs with few very small teeth. otherwise foliage as scopulorum. Riparian woodland. limestone. male tree, ca. 10 cm dbh, 3 m tall; bark longitudinally plated. Associated species: *Pinus ponderosa var. brachyptera, Pinus edulis, Juniperus deppeana, Quercus muehlenbergii, Quercus grisea, Acer grandidentatum, Berberis haematocarpa, Arbutus xalapensis var. texana Dasylirion leiophyllum, Agave parryi,* Lincoln National Forest, Guadalupe Mountains, Dark Canyon, S of Klondike Gap near confluence Hooper Canyon, 0.2 mi (by FR 307) E jct County Road 412 (FR 69), just inside USFS boundary. TRS: T25S R21E sec 26 SE1/4, 32° 6' 0" N, 104° 46' 15.6" W. 1920m. (6300 ft.), 3 November 2019, **Eddy County, NM**
Lab Acc. *Robert P. Adams 15804*, *ex* Coll. *George M. Ferguson 4658,*
scale lvs with few very small teeth. otherwise foliage as scopulorum. male tree, 90 cm dbh, 12 m tall; bark rough longitudinally plated, reddish brown beneath, few old pollen cones falling, no new cones yet, Lincoln National Forest, Guadalupe Mtns., upper Devil's Den canyon, on limestone, Pinyon-oak-juniper woodland w *J. deppeana* and *Pinus* sp. T26S, R21E, Sec 16 SE ¼ , 32° 2' 17.16" N, 104° 48' 2.52" W, 2182m (7155 ft), 19 Jan 2020, Eddy County, NM

Voucher specimens are deposited at the Herbarium, Baylor University (BAYLU).

Isolation of Oils- Fresh leaves (200 g) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

Volatile oil Analyses- Oils from 10-15 trees of each of the taxa were analyzed and average values are reported. The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details, out of print, free pdf: www.juniperus.org). Identifications were made by library searches of our volatile oil library (Adams, 2007, www.juniperus.org), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

DNA analysis - One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM), (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS (ITS+42F, ITS-57R) and petN-psbM (petN5F, psbM111R) primers utilized. Due to the presence of an indel (1 bp deletion at site 194 in *J. scopulorum*), sequences for the mon x scop hybrids were not readable from site 194 forward. A reverse primer was designed (ITS765r, ATC GCA CTT CAT TCT TTT Tm 49.7˚C) and then synthesized by IDT (Integrated DNA Technologies, Inc.), San Diego, CA. This primer was used to obtain clean sequences in hybrids for sites S2-S16. The reverse primer, ITS-57R, was to read site S25.

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

RESULTS AND DISCUSSION

Sequencing nrDNA (ITS) resulted in 1270 bp and comparison between *J. monosperma* and *J. scopulorum* revealed 25 SNPs plus 5 indels. Due the difficulty of sequencing sites 18 - 24, these sites were not analyzed. These large number of differences between *J. monosperma* and *J. scopulorum* underscore the magnitude of phylogenetic differences between the serrate leaf junipers and the smooth (entire) leaf margined junipers. In fact, these clades are thought to have migrated to North America (NA) at different times, and by different routes. The serrate leaf junipers appear to have migrated to NA ca. 47 to 30.5 Mya from Europe via the NALB (North America Land Bridge) from Europe to Iceland, Greenland, Nova Scotia, thence into the dry Madrean -Tethyan vegetation zones in southwestern US and Mexico (see Fig. 1.4, Adams 2014). The smooth leaf margined junipers are very closely related to *J. sabina*/ *J. davurica* in the China - eastern Russian area of the eastern hemisphere, thence across the BLB (Bering Land Bridge) ca. 17.6 to 5.5 Mya (see Fig. 1.7, Adams 2014).

The ITS sequences clearly revealed that 3 unusual plants, appearing to be a variant of *J. scopulorum* in the field, were heterozygous for the 18 ITS variable sites (Table 1). These 3 plants (15601, 15787 and 15804, appearing morphologically as *J. scopulorum* in the field, are hybrids by their ITS DNA.

Table 1. SNPs from nrDNA(ITS) and cp DNA classification of *J. scopulorum, J. monosperma* and *J. monosperma* x *J. scopulorum* from Guadalupe Mtns., NM.na = not available.

Acc. # &	pollen pat.	nuc. mat.	nrDNA (ITS) 18 of 25 informative sites[1]																
field id species	cp	ITS	S1 179	S2 205	S3 257	S4 285	S5 315	S6 338	S7 348	S8 350	S9 352	S10 353	S11 368	S12 406	S13 422	S14 432	S15 545	S16 614	S17 615	S25 1173
15602 scop	scop	scop	C	C	C	T	A	C	T	T	G	T	C	G	T	A	T	C	T	T
15603scop	scop	scop	C	C	C	T	A	C	T	T	G	T	C	G	T	A	T	C	T	T
15783 scop	scop	scop	C	C	C	T	A	C	T	T	G	T	C	G	T	A	T	C	T	T
15799 scop	scop	scop	C	C	C	T	A	C	T	T	G	T	C	G	T	A	T	C	T	T
15601 scop	mon	MxS	C/T	na	na	C/T	A/T	C/T	G/T	C/T	A/G	C/T	C/T	A/G	C/T	A/G	G/T	C/T	G/T	na
15787scop	mon	MxS	C/T	C/T	C/T	C/T	A/T	C/T	G/T	C/T	A/G	C/T	C/T	A/G	C/T	A/G	G/T	C/T	G/T	C/T
15804scop	mon	MxS	C/T	C/T	C/T	C/T	A/T	C/T	G/T	C/T	A/G	C/T	C/T	A/G	C/T	A/G	G/T	C/T	G/T	C/T
15781 mon	mon	mon	T	T	T	C	T	T	G	C	A	C	T	A	C	G	G	T	G	C
15807 mon	mon	mon	T	T	T	C	T	T	G	C	A	C	T	A	C	G	G	T	G	C
15782 mon	mon	mon	T	T	T	C	T	T	G	C	A	C	T	A	C	G	G	T	G	C
15805 mon	mon	mon	T	T	T	C	T	T	G	C	A	C	T	A	C	G	G	T	G	C
15806 mon	mon	mon	T	T	T	C	T	T	G	C	A	C	T	A	C	G	G	T	G	C

[1]S1,179:xGCGGACA,S2,205:xGCTGGAGGG; S3,257:xGAATGCCG; S4,285: xCCCGCGG; S5,315: xTCTGGATC;S6, 338: xCGAAACGA; S7,348: CGAAACGAx; S8,350(y): CGAAACGAxTy; s9,352(z), S10,353(!): CGAAACGAxTyTz!;S11,368:xCCCTGCTC; S12,406: xTCCCCCGT; S13,422:xCATGGCTC; S14, 432: xTCGTGTGC; S15,545: xTGTTCAGG;S16,614: CTCTCCCTx; S17,615(y): CTCTCCCTxy; S25, 1173: xGCGGGCA;

Sequencing petN-psbM yielded 5 informative SNPs that resolved the *J. monosperma* cp from *J. scopulorum* cp. Thus, all samples could be readily scores as having *J. monosperma* or *J.* scopulorum cp (Table 1). The three tress that were field identified, morphologically, as '*J. scopulorum*', were all hybrids in their nrDNA, and each had the *J. monosperma* cp (Table 1).

Examination of the leaf margins (40x) revealed they were smooth, except for very small teeth near the bottom of the leaf margins. Plant 15601 specimen did not have seed cones and plants 15787 and 15804 were males, so no seed cones were available to observe.

If these 3 plants are indeed F_1 hybrids, it seems odd that the morphology is so similar to*J. scopulorum*. So, we decided to investigate the leaf essential oils as they have proven useful to detect hybridization in *Juniperus* (Flake et al. 1978; Adams 1983, Palma-Otal et al. 1983; Adams and Kistler 1991, Adams 2013a, b).

The volatiles oils of *J. monosperma* and *J. scopulorum* have been published (RPA) from several locations from our (RPA) lab: *J. monosperma* (Adams 1994; Adams et al. 2014a, 2014b) and *J. scopulorum* (Adams 2009, 2015a). However, it is important to analyze oils from trees in the vicinity of the putative hybrids. The volatile leaf essential oil (EO) of *J. monosperma* is dominated by α-pinene (62.3 - 75.8%), with moderate amounts of β-phellandrene (5.3-7.1%), elemol (0.7 - 3.1%), β-eudesmol (0.9 - 8.4%) and 8-α-acetoxyelemol (0.8 - 1.0%). The EO of *J. scopulorum* is dominated by sabinene (40.7 - 48.4), with moderate amounts of α-pinene (2.6 - 2.7%), limonene (2.1 - 1.8%), β-phellandrene (1.7), terpinen-4-ol (1.7 - 3.7%),pregeijerene B (6.3 - 8.7%), germacrene D-4-ol (1.6 - 1.7%) and 8-α-acetoxyelemol (4.5 - 4.9%).

The EO of *J. monosperma* and *J. scopulorum* differ distinctly (Table 2) in 15 compounds (bold face): α-thujene, α-pinene, sabinene, β-pinene, α-terpinene, limonene, β-phellandrene, camphor, coahuilensol, terpinen-4-ol, pregeijerene B, germacrene B, germacrene D-4-ol, α-cadinol and 8-α-acetoxyelemol. Often, the concentrations of EO components is intermediate between parents of hybrids (Adams and Tsumura 2012; Adams and Stoehr 2013), and this is the case for 10 compounds: α-thujene, α-pinene, sabinene, β-pinene, γ-terpinene, cis-sabinene hydrate, trans-sabinene hydrate, terpinen-4-ol, germacrene D-4-ol, and 8-α-acetoxyelemol (Table 2). These data provide strong support that the unusual plants (15797, 15804) are hybrids. It might be noted that the two *J. scopulorum* EO (15783, 15799, Table 2) represent the two chemotypes present in *J. scopulorum* (and *J. virginiana*). This appears to be due to a single gene that appears to turn on the production of aromatic ethers synthesized in the phenylpropanoid pathway that is separate from the terpenoid pathway (von Rudloff 1975; Adams et al. 1981). Note the presence of safrole, methyl eugenol, (Z)-isoeugenol, and elemicin (scop 15804, Table 2), which were co-extracted in the terpenoids. It appears a high aromatic ethers type plant (cf. 15804) is not a parent of the hybrids (15787 and 15804) because they are both devoid of aromatic ethers (i.e., safrole, methyl eugenol, (Z)-isoeugenol, and elemicin).

Ten of the EO components are transgressive (i.e., concentration of a compound it larger (or smaller) than the concentration in either parent). Nine transgressive compounds have a higher concentration in the hybrids than in either parent:α-fenchene, δ-2-carene, δ-3-carene, limonene, β-phellandrene, terpinolene, cis-p-menth-2-en-1-ol, trans-p-menth-2-en-1-ol, α-terpineol and abietadiene (Table 2). Only one, pregeijerene B has a lower concentration than either parent (Table 2). Analyses of the inheritance of terpenoids in this study versus in *Cryptomeria japonica* and *Pseudotsuga menziesii*, reveals that intermediate inheritance (10 cpds., this study) is comparable (Table 3) to *C. japonica* (7 cpds.) and *P. menziesii* (11, 2 plus 8 dominant or recessive cpds.). The number of transgressive cpds. (higher conc.) in this study (10) is similar to *C. japonica* (5 cpds.) and *P. menziesii* (9, 4) and the number of transgressive cpds. (lower conc.) in this study (1) is low compared to *C. japonica* (5 cpds.) and *P. menziesii* (5,5).

Another facet of mixing germplasms in hybrids genomes is that some biochemical pathways can produce novel (new) compounds because the enzymes from both parents may be present in a synthesis region in the cell. For example, an acetylation enzyme from one parent may act upon α-terpineol to produce α-terpinyl acetate as seen in Table 2 (both parents have α-terpineol but not α-terpinyl acetate, as it is only found in the hybrids). The hybrids had 18 "new" compounds NOT found in either parent (Table 2, 3)! This compares to one (1) in *C. japonica* and none (0) in *P. menziesii*. The hybrids analyzed from *C. japonica* and *P. menziesii* (Adams and Tsumura2012; Adams and Stoehr 2013) were all derived from infra-specific crosses in which the genomes were very similar. Thus, no new compounds resulted from those crosses.

Table 3. Inheritance of terpenoids in hybrids in this study compared with inheritance in literature reports.

Mode of inheritance in hybrids vs. number of compounds	This study	*Cryptomeria japonica* (Adams and Tsumura (2012)	*Pseudotsuga menziesii* (Adams and Stoehr 2013)	
			wide cross	narrow cross
Concentration intermediate between *monosperma* and *scopulorum* plants sampled.	10	7	11	2 (+ 8 dominant/ recessive)
Transgressive, higher conc. than found in *monosperma* or *scopulorum* plants sampled.	10	5	9+ % oil yield	4 + % oil yield
Transgressive, lower conc. than found in either *monosperma* or *scopulorum* plants sampled.	1	5	5	5
Novel cpds. not found in either *monosperma* or *scopulorum* plants sampled.	18	1	0	0

The distribution of *Juniperus monosperma* in the Guadalupe Mts. is from the north, where it occurs at mid-elevations of the adjacent Sacramento Mts., NM, southward along The Rim in the northern portion of the Guadalupe Mts., predominately at 5800 – 6200 ft. It grows in a low-profile pinyon-juniper woodland with the associated *Juniperus deppeana*, *Pinus edulis* and *Quercus grisea*. Outlying plants extend onto the base of the western escarpment with scattered individuals to ca. 5000 ft., in semidesert grassland and a few plants as low as 4400 ft. in Chihuahuan desert-scrub. Apparently, *J. monosperma* is rare in the canyons or pediment of the southern escarpment of the Guadalupe Mts. (in Texas) although populations extend farther south to the adjacent Diablo and Apache Mts. Whereas *J. monosperma* is tolerant of xeric environments, the more mesic habitat requirements of *Juniperus scopulorum* limit it to riparian canyon bottoms and north-facing slopes of canyons. The southernmost population for the species is in the Guadalupe Mts., where the distribution of *J. scopulorum* is disjunct from the upper portions of the Sacramento Mts. to the north. In the Guadalupe Mts., *J. scopulorum* occurs at upper elevations, primarily at 6300-7200 ft., in riparian woodlands in the north-central portion of the Guadalupe Mts., while some individuals extend down into the largest canyons of the southern escarpment (in McKittrick canyon, Texas) to ca. 6000 ft. with the associated *Juniperus deppeana*, *Pinus*, *Quercus*, *Acer* and *Arbutus*.

It appears that *J. scopulorum* and *J. monosperma* are separated elevationally, and by habitat such that locally, the species are essentially allopatric within the Guadalupe Mts. In other regions across their ranges, which are widely overlapping, these two species can be locally sympatric (e.g. Gila National Forest, NM, and Mogollon Rim, AZ). From our observations, the nearest *J. monosperma* to the hybrid *J. scopulorum* tree in Dark Canyon at 6300 ft., is 3.5 air miles N near the jct. of NM 137 and FR 520 at 6100 ft., or 3.5 mi ENE in lower in Dark Canyon at 5800 ft., or 4.0 air miles WNW on NM 137 at 5800 ft. The *J. monosperma* nearest to the two hybrid *J. scopulorum* trees in Devil's Den Canyon at 7150 ft., is 4.5 air miles NNE below The Rim near El Paso Gap at 5450 ft., or 5.0 air miles NNE on NM 137 at 5800 ft. The sparse juniper-oak-pine woodland in upper Dog Canyon (ca. 2 air miles W of the hybrid trees, at the mouth of Devil's Den Canyon) is apparently *J. deppeana* without *J. monosperma* at 6100-6200 ft. However, this general discussion does not eliminate the possibility that a few scattered *J. monosperma* trees may occur nearer the hybrids.

Inasmuch as *J. deppeana* is widespread in the Guadalupe Mts., and occurs at a wide range of elevations (5500 – 8000 ft.) and, thus, is sympatric with *J. scopulorum* and *J. monosperma*, these latter occur in different, specific habitats. Along the southern pediment and lower eastern slopes of the

Fig. 1(left). Putative mono x scop hybrid tree with round crown shape (RPA 15804, GF4658). Note the the several large side branches and irregularly spaced, odd angled and balls of foliage on branches.

Fig. 1(right) Typical crown of *J. scopulorum*. Note the strong central axis, pyramidal shape, and the regularly spaced, uplifted side branches.

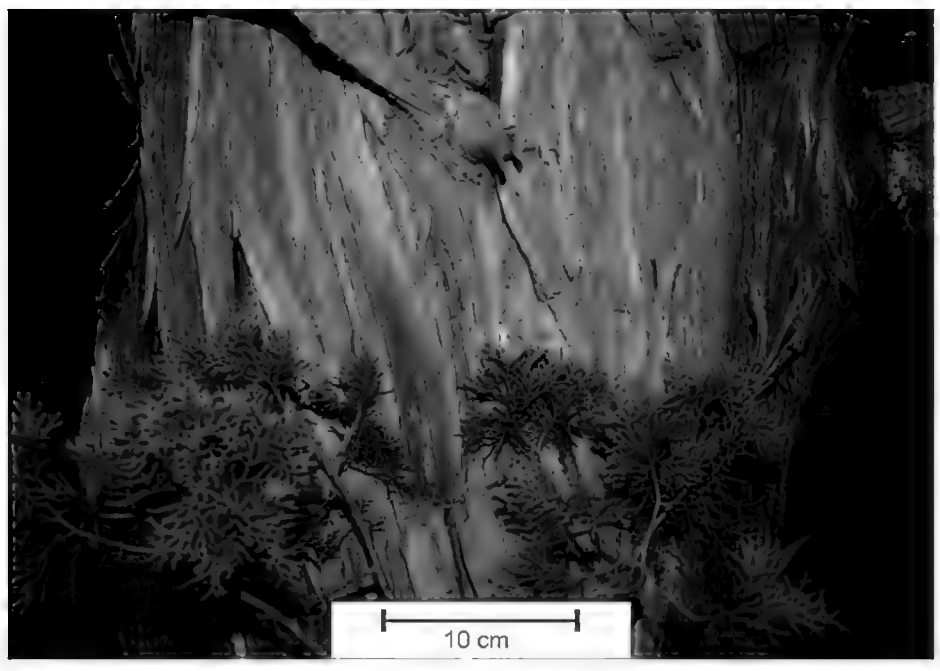

Figure 2 (left). Leaves and bark of mono x scop hybrid tree, Lab 15804 (GF4658). Note dark green leaves and short, strong branchlets.

Fig. 2 (right). Leaves and bark of scopulorum, GF 4649. Note glaucous leaves and longer branchlets.

Guadalupe Escarpment, *Juniperus pinchotii* is also present, though generally not sympatric with either *J. scopulorum* or *J. monosperma*. Additionally, *J. pinchotii* sheds its pollen in the fall prior to November (Ferguson personal observation; Adams 2014), in contrast the other junipers in the area, pollen is shed is in the spring (Adams 2014). Comparison of the putative hybrid, (RPA 15804, GF4658) (Fig. 1,left) with typical J. scopulorum (Fig. 1, right) reveals that the hybrid has a round crown (as do the branch tips) vs. the pyramidal crown, and elongated branch tips. In addition, the hybrid has several large side branches compared to fairly uniform and equally spaced, uplifting side branches in *J. scopulorum*. The foliage of the hybrid is more compact, and greener that that of *J. scopulorum* (Fig. 2, left vs. right). The bark of the hybrid is twisted and exfoliation in very thick strips vs. thinner strips in *J. scopulorum* (Fig. 2, left vs. Taken together, EO components being intermediate, transgressive and newly found, DNA complementary in the hybrid, and morphology, these provide strong evidence that the putative hybrids are indeed hybrids between *J. monosperma* and *J. scopulorum.* Additional research is needed to more fully understand this evolutionary event.

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Table 2. Compositions of the leaf oils of *J. monosperma* (mono), *and J. scopulorum* (scop) and putative hybrids. Green highlight = intermediate concentration between *monosperma* and *scopulorum*; Yellow = transgressively larger concentration than either *monosperma* or *scopulorum*; Tan = transgressively smaller in hybrids than in putative parents; Blue = cpd. not found in either putative parent species, Compounds that appear to separate the parents are in boldface. Aromatic ethers (only found in 15799) are in purple.

KI	compound	mono 15781	mono 15807	mono x scop 15787	mono x scop 15804	scop 15783	scop 15799
921	tricyclene	t	t	t	t	t	t
924	**α-thujene**	**t**	**t**	**0.9**	**0.6**	**1.6**	**1.1**
932	**α-pinene**	**62.3**	**75.8**	**21.5**	**13.2**	**2.7**	**2.6**
945	**α-fenchene**	**t**	**t**	**0.5**	**0.6**	**t**	**t**
946	camphene	0.3	t	t	t	t	t
953	thuja-2,4(10) diene	t	t	-	-	-	-
969	**sabinene**	**0.1**	**0.2**	**23.1**	**18.6**	**48.4**	**40.7**
974	**β-pinene**	**1.2**	**1.8**	**0.8**	**0.9**	**t**	**t**
988	myrcene	0.7	1.6	3.0	3.4	1.5	0.8
1001	δ-2-carene	t	t	5.1	0.2	0.2	t
1002	α -phellandrene	0.4	0.5	t	0.3	t	t
1008	δ-3-carene	0.3	t	7.9	15.0	t	t
1014	**α -terpinene**	**t**	**t**	**0.8**	**0.6**	**1.4**	**0.8**
1020	p-cymene	0.3	0.3	0.5	0.9	0.3	0.1
1024	**limonene**	**-**	**-**	**4.6**	**1.8**	**2.1**	**1.8**
1025	**β-phellandrene**	**5.1**	**7.1**	**4.5**	**13.4**	**1.7**	**1.7**
1132	limonene oxide	-	-	-	**0.3**	-	-
1044	(E)-β-ocimene	t	t	0.2	0.2	t	t
1054	γ-terpinene	0.5	0.5	1.4	1.0	2.3	1.4
1065	cis-sabinene hydrate	t	t	0.6	0.6	1.3	0.7
1086	terpinolene	0.7	0.9	1.5	1.9	1.3	0.8
1097	trans-sabinene hydrate			0.5	0.3	1.2	0.6
1097	linalool	0.3	t	0.4	0.3	-	-
1100	n-nonanal	t	t	-	-	-	-
1101	cis-thujone (= α-thujone)	-	-	-	-	-	t
1108	1,3,8-p-menthatriene	-	-	-	-	t	-
1112	trans-thujone (= β-thujone)	-	-	-	-	t	t
1118	cis-p-menth-2-en-1-ol	t	t	**0.4**	**0.5**	0.3	0.2
1122	α-campholenal	t	t	t	t	-	-
1136	**trans-p-menth-2-en-1-ol**	t	t	**0.3**	**0.3**	0.1	t
1138	gejgerene	-	-	-	-	t	t
1140	trans-verbenol	-	-	**t**	-	-	-
1141	**camphor**	**0.3**	**0.2**	-	**0.2**	-	-
1145	**camphene hydrate**	**0.1**	t	-	-	-	-
1158	trans-pinocamphone	t	t	-	-	-	-
1165	borneol	t	t	-	-	t	t
1166	p-mentha-1,5-dien-8-ol	-	-	0.2	0.3	-	-
1066	**coahuilensol**	**t**	**0.1**	**-**	**-**	**-**	**-**
1174	**terpinen-4-ol**	**0.2**	**0.2**	**2.2**	**1.7**	**3.7**	**1.7**
1183	cryptone	-	-	-	**0.3**	-	-
1189	p-cymen-8-ol	t	t	t	t	t	t
1186	α-terpineol	0.1	0.1	0.3	0.3	0.2	t
1195	methyl chavicol	t	-	-	0.3	-	t

KI	compound	mono 15781	mono 15807	mono x scop 15787	mono x scop 15804	scop 15783	scop 15799
1195	cis-piperitol	-	-	t	t	t	t
1198	methyl salicylate	t	0.1	-	-	-	-
1199	safranal	-	-	0.1	-	-	-
1204	verbenone	-	-	-	**0.3**	-	-
1207	trans-piperitol	-	-	t	t	t	t
1219	**coahuilensol, methyl ether**	t	**t**	-	-	-	-
1223	citronellol	-	-	t	0.3	-	0.2
1232	thymol, methyl ether	-	-	1.1	t	-	-
1235	**trans-chrysanthenyl acetate**	**0.2**	**0.1**	-	-	-	-
1239	carvone	t	t	-	-	-	-
1249	piperitone	0.2	t	-	-	t	-
1254	linalyl acetate	-	-	0.1	t	-	-
1274	**pregeijerene B**	**2.0**	**2.7**	**0.6**	**1.2**	**6.3**	**8.7**
1285	safrole	-	-	-	-	-	**15.5**
1287	bornyl acetate	0.6	0.6	0.3	t	0.2	t
1289	thymol	-	-	0.2	t	-	-
1315	(2E,4E)-decadienal	t	t	t	t	t	t
1345	α-terpinyl acetate	-	-	**0.3**	**t**	-	-
1345	α-cubebene	-	-	-	-	-	t
1374	α-copaene	-	-	-	-	t	-
1396	duvalene acetate	t	0.1	t	t	-	-
1391	(2E,4Z)-methyl decadienoate	-	-	t	-	t	-
1403	methyl eugenol	-	-	-	-	t	**2.5**
1407	longifolene	-	-	-	**t**	-	-
1417	(E)-caryophyllene	t	t	0.3	-	0.3	0.1
1451	(Z)-methyl isoeugenol	-	-	-	-	-	0.3
1451	trans-muurola-3,5-diene	-	-	-	-	0.3	-
1452	α-humulene	0.2	t	0.4	-	t	t
1465	cis-muurola-3,5-diene	-	-	-	-	t	t
1468	**pinchotene acetate**	t	**t**	-	-	-	-
1475	trans-cadina-1(6),4-diene	-	-	-	-	t	t
1480	germacrene D	-	-	0.2	-	0.7	0.3
1493	trans-muurola-4(14), 5-diene	-	-	-	-	**0.2**	t
1493	epi-cubebol	-	-	-	-	-	0.1
1500	α-muurolene	0.2	0.2	t	t	0.5	0.3
1513	γ-cadinene	-	-	t	t	1.1	0.5
1521	trans-calamenene	-	-	-	**0.2**		
1522	δ-cadinene	-	-	t	0.3	1.7	1.1
1537	α-cadinene	-	-	-	-	0.1	t
1539	α-copaen-11-ol	t	t	-	0.1	-	0.1
1549	elemol	3.1	0.7	4.7	2.4	5.0	3.9
1555	elemicin	-	-	-	-	-	1.2
1559	**germacrene B**	**0.5**	**0.1**	-	-	-	-
1574	**germacrene D-4-ol**	-	-	**0.5**	**1.4**	**1.6**	**1.7**
1582	caryophyllene oxide	-	-	0.3	-	-	-
1594	ethyl decanoate	-	-	0.2	-	-	-
1607	β-oplopenone	-	-	-	0.2	0.3	0.2
1608	humulene epoxide II	-	-	0.4	-	-	-
1630	γ-eudesmol	2.2	0.4	0.4	0.3	0.4	0.2
1638	epi-α-cadinol	-	-	t	0.2	0.4	0.3
1638	epi-α-muurolol	-	-	t	0.3	0.5	0.2

KI	compound	mono 15781	mono 15807	mono x scop 15787	mono x scop 15804	scop 15783	scop 15799
1644	α-muurolol	-	-	t	t	t	t
1649	β-eudesmol	8.4	0.9	0.9	0.4	0.8	0.3
1652	α-eudesmol	1.4	0.6	0.6	0.5	0.8	0.4
1653	**α-cadinol**	**-**	**-**	**0.5**	**0.4**	**0.6**	**0.5**
1792	**8-α-acetoxyelemol**	**0.8**	**1.0**	**2.2**	**3.9**	**4.9**	**4.5**
1887	oplopanonyl acetate	-	-	-	**0.1**	-	-
1933	cyclohexadecanolide	-	-	-	**0.2**	-	-
1959	hexadecanoic acid	0.6	0.5	0.2	0.1	-	-
2009	manool oxide	t	t	-	-	-	-
2055	abietatriene	t	t	t	0.1	t	t
2087	abietadiene	t	t	0.4	0.2	t	t
2298	4-epi-abietal	-	-	0.4	0.2	0.2	0.1
2312	abieta-7,13-dien-3-one	-	-	-	0.3	t	t
2313	abietal	-	-	-	0.3	-	-
2314	trans-totarol	0.2	t	1.3	-	-	-
2331	trans-ferruginol	0.1	t	0.2	0.1	-	-
2343	4-epi-abietol	t	-	0.2	t	t	t
2401	abietol	-	-	t	t	t	t
2443	methyl neo-abietate	t	t	-	-	-	-

KI = Kovat's Index (linear by temperature programming) om J & W DB-5 column. Values less than 0.05% are denoted as traces (t). Unidentified components less than 0.5% are not reported.

The effects extreme alkaline soil on biomass and hydrocarbon yields in *Helianthus annuus* cv. Munchkin, Firecracker and Little Becka (Asteraceae, Sunflowers)

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ABSTRACT

Sunflowers, *H. annuus* cv. Munchkin, Firecracker and Little Becka, were greenhouse grown in alkaline soil, common in St. George, UT, to determine its effects on leaf biomass, percent yields of free hydrocarbons (HC), and yields of gHC/ g biomass. Biomass was very reduced, but was 40 - 60% larger for Firecracker and Little Becka than for Munchkin. Percent (%) hydrocarbon (HC) yield was largest in Little Becka (4.14%). with Firecracker and Munchkin having significantly lower yields (3.47 - 3.16%). Yields as g HC/ g 10 leaves were very small with only 0.05 - 0.06 g for Little Becka and Firecracker and only 0.03g for Munchkin. Comparison with a previous study of Munchkin revealed the biomass, % HC yield, and gHC/ g 10 leaves in Munchkin grown in alkaline soil was far lower than even plants grown in potting soil watered with 120 mM salt or 240 mM salt solutions. In contrast to salt (NaCl) stress, alkaline stress did not result in an increase of HC yields. Published on-line www.phytologia.org *Phytologia 102(3): 143-149 (Sept 21, 2020).* ISSN 030319430.
KEY WORDS: *Helianthus annuus,* Sunflower, alkaline soil effects on hydrocarbon yields.

A recent study examined the effects of watering using increasing concentrations of salt and pure sand on HC (hydrocarbon) yields and biomass (Johnson, Theobald and Adams 2019). They found biomass, % HC yields, and g HC yields all declined, from the control, to 30 mM, and 120 mM salt (red ellipses, Fig. 1); however, at 240 mM salt, the % HC yield increased (Fig. 1). An unusual trend was seen in the sand treatment (nutrient deficiency) where Munchkin % HC yield increased, but its biomass was very low (Fig. 1). The total g HC yields were not significantly different among the wide range of salt concentrations utilized in that study (yellow ellipse, Fig. 1). Considering that seawater is approximately 600 mM salinity, the 240 mM salt water is a very high salt concentration (~ 40% of seawater). Although less biomass would be produced, total g HC yields (yellow ellipse, Fig. 1) were not significantly different, even with increasingly higher salt concentrations.

Defense chemicals are both constitutive and inducible defenses (see Whittstock and Gershenzon, 2002 for discussion). Recently, we reported (Adams et al. 2017c) that progeny of high hydrocarbon (HC) yielding sunflower (*H. annuus*) populations displayed much reduced HC yields when grown in greenhouse conditions. We reported the percent HC (greenhouse / field grown HC yields) decreased to 45.9, 55.6 and 78.3%. In addition, g HC / g DW weights of leaves were very reduced to from 17.9 g to 6.1 g when plants were grown in a greenhouse. It appears that biotic and abiotic factors in natural populations can have large effects on HC yields.

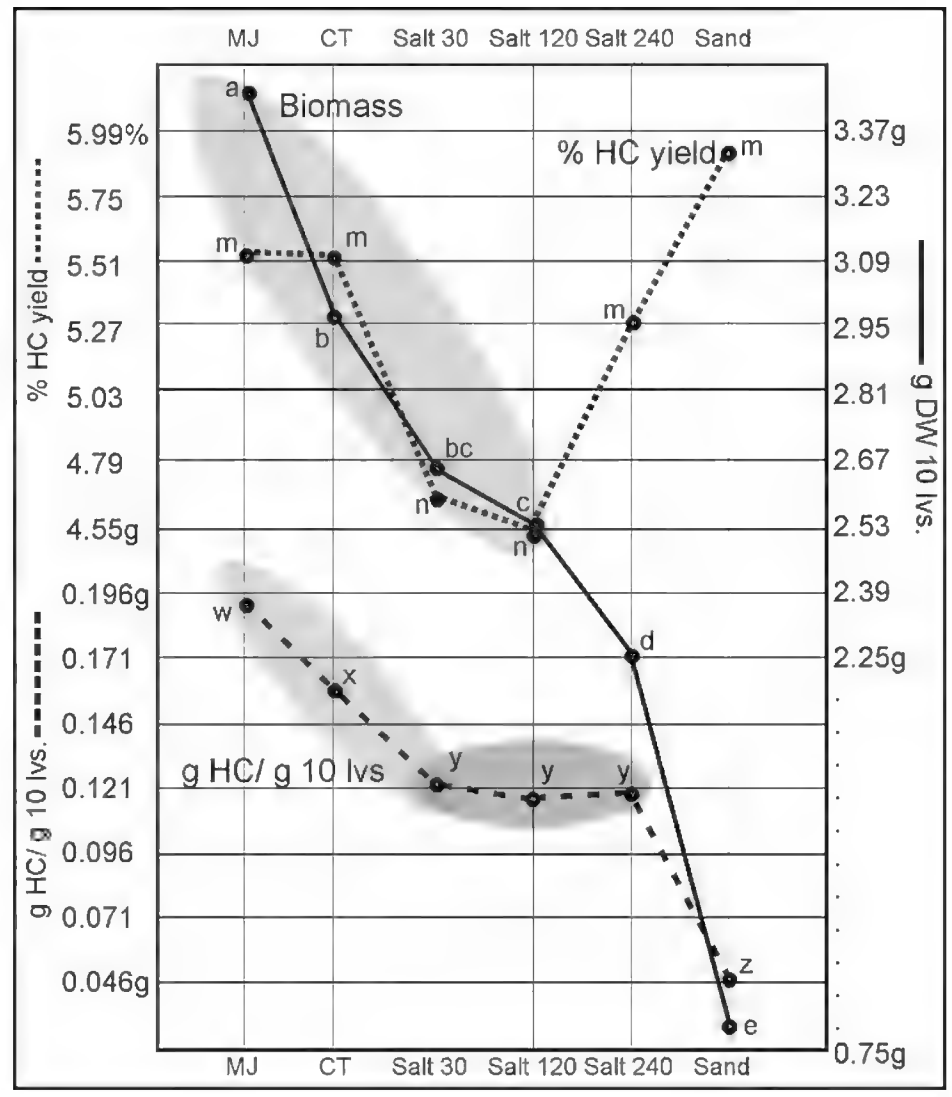

Figure 1. Graphs of dry weight (10 leaves), percent HC yields, and g HC/ g DW 10 leaves for Munchkin subjected to 5 treatments. Means with the same letter superscripts are **not** significantly different (P= 0.05). Similar trends are noted by the red ellipses. The yellow ellipse highlights the uniform HC yields from plants watered with increasing salt concentrations (30 mM, 120 mM, 240 mM) of saltwater. See text for discussion.

The purpose of the present paper is to report the effects nutrient-poor alkaline soil, typical of St. George, Utah, on biomass, % HC yields and gHC/ g DW leaves for 3 sunflower cultivars: Munchkin, Little Becka and Firecracker (*Helianthus annuus* cultivars). This report is a part of a continuing study on the development of sunflowers as a source for natural rubber and bio-fuels from the biomass (Adams et al., 1986; Adams and Seiler, 1984; Adams and TeBeest, 2016; Adams et al. 2016; Adams and TeBeest, 2017; Adams et al. 2017a,b,c; Adams and Johnson 2018; Adams et al. 2018a,b,c; Johnson et al. 2019; Pearson et al., 2010a,b; Seiler, Carr and Bagby, 1991,).

MATERIALS AND METHODS

Seeds of *H. annuus* cv. Munchkin , Firecracker and Little Becka were obtained from Sunflower Selections, Inc., Woodland, CA. Seeds were germinated in potting soil in 2" square cups in a lab growth chamber, then one week after germination they were transplanted into 6 " square plastic pots using two kinds of alkaline soils from St. George, UT. Soil testing was by A & L labs, Modesto, CA. Two sets of 42 plants (14 each of Munchkin, Firecracker and Little Becka) were grown in the greenhouse at Pine View High School (PVHS), St. George, UT from Feb. 15, 2020 until March 15, 2020 when the CovID-19 virus led to closing the school. The plants were then transferred to a growth chamber with LED lighting approximately equal to daylight for 16 hr light, 8 hr dark cycles. Plants were watered with 200 ml tap water twice per week in the PVHS greenhouse. After moving to the lab growth chamber, plants were watered when wilted leaves appeared. The 10 largest, non-yellowed, mature leaves were collected. The leaves were air dried in paper bags at 49° C in a plant dryer for 24 hr or until 7% moisture was attained. Leaves were ground in a coffee mill (1mm). 3 g (or less in some cases) of air-dried material (7% moisture) were placed in a 125 ml, screw cap jar with 20 ml hexane, the jar was sealed, then placed on an orbital shaker for 18 hr. The hexane soluble extract was filtered through a Whatman (P8) filter paper into a pre-weighed aluminum pan and the hexane evaporated on a hot plate (50°C) in a hood. The pre-weighed aluminum pan with concentrated hydrocarbon extract was weighed and tared. Extraction of identical samples by shaking and soxhlet (8 hr) yielded a correction factor of 1.9 (soxhlet yield/ shaking yield), which when corrected to

oven dry weight basis (ODW) by 1.085 resulted in a total correction factor of 2.06. ANOVA and SNK (Student Newman-Keuls) multiple range tests were programmed following the formulations in Steel and Torrie (1960).

RESULTS

Table 1 shows the results from the treatments ANOVA and SNK statistical analyses. Biomass was highly significantly larger in Little Becka and Firecracker, than in Munchkin (Table 1, Fig. 2). Previously, we found (Johnson et al. 2019) biomass from Munchkin grown in potting soil is about 3g (Fig. 1) vs. 0.965 g in this study (Table 1). Percent (%) HC yields were significantly higher in Little Becka (4.14%) (Table 1). Yield of HC (as gHC/ g DW 10 leaves) was significantly higher in Firecracker and Little Becka, but, of course, very small due to the small amount of biomass (Table 1,Fig. 2).

Table 1. Comparison of dry weight (10 leaves), in % HC yields, and g HC/ g DW 10 leaves for cv. Munchkin, Firecracker and Little Becka grown on alkaline soil. Mean values with the **same suffix letter** (gray highlighted) are **not** significantly different (P= 0.05).

	Munchkin	Little Becka	Firecracker	F ratio, significance
Biomass, g DW 10 leaves	0.965a	1.41b	1.55b	F= 6.104, P = 0.007 ***
	Munchkin	Firecracker	Little Becka	F ratio, significance
% HC yield	3.16 e	3.47e	4.14f	F= 9.098, P = 0.00125***
	Munchkin	Firecracker	Little Becka	F ratio, significance
g HC/ g DW 10 leaves	0.030y	0.053z	0.058z	F= 9.683, P = 0.946***

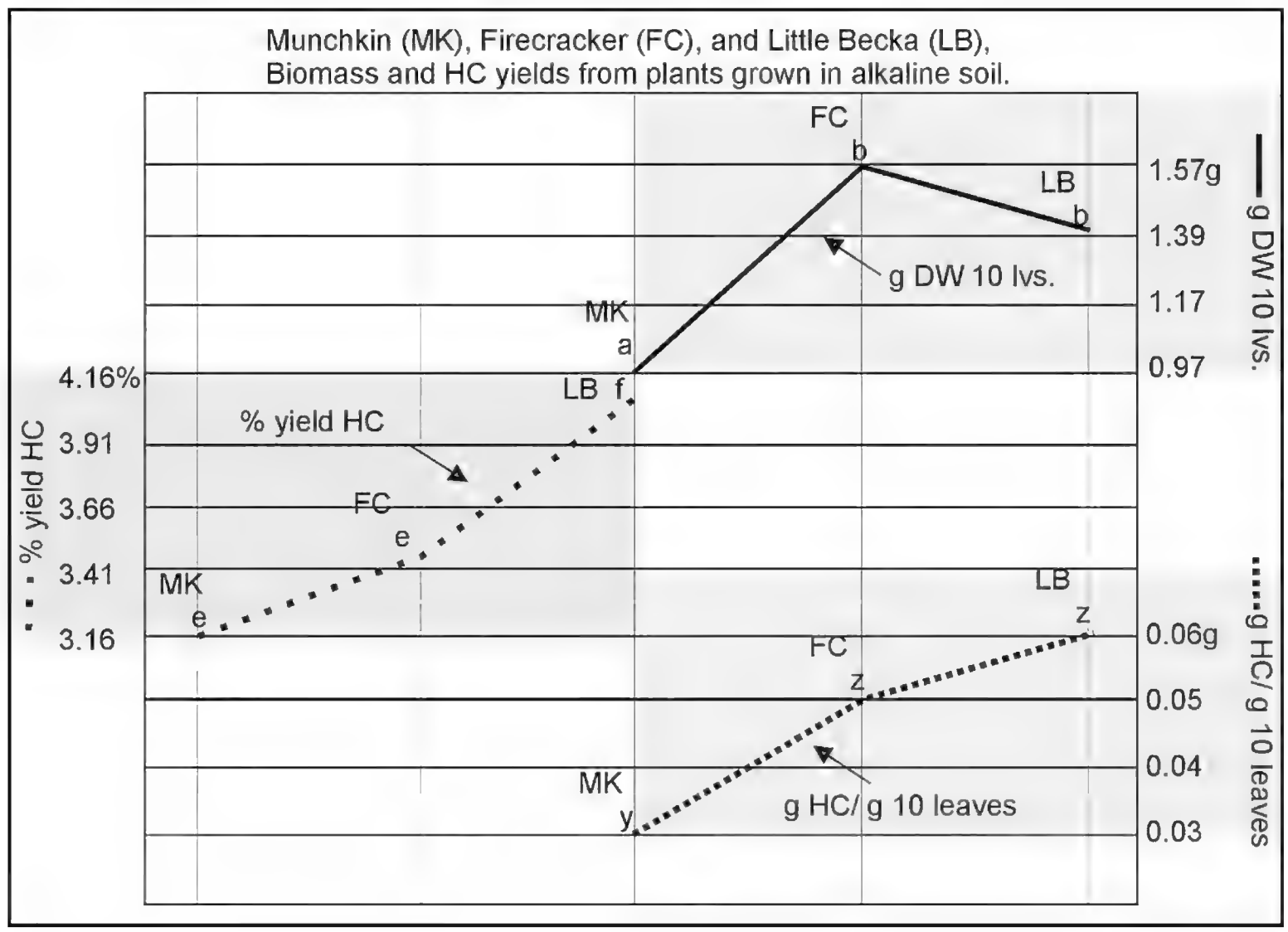

Figure 2. Graphs of biomass, % HC yield, and g HC/ g DW 10 leaves for Munchkin, Firecracker and Little Becka plants grown in alkaline soil.

Figure 3. Little Becka, Munchkin, and Firecracker in bud/ flower at time of harvest in alkaline soil.

Munchkin is a very dwarf sunflower cultivar and this is shown in Figure 3. Firecracker and Little Becka had the largest leaf biomass. In summary, it is clear that Little Becka and Firecracker grow better and produce higher yields of HC in St. George, alkaline soil than Munchkin.

Of the 42 plants of Munchkin, Little Becka and Firecracker grown in very alkaline soil, only one Firecracker plant survived (Fig. 4). The **entire** dry weight (stem, leaves and roots) of the plant (Fig. 4) was only 0.06 g! It consisted of 4 leaves (shown in Fig. 4). One can see 3 aborted leaf scars on the stem above the 4 leaves (Fig. 4). Generally, after 2 or 4 leaves were produced, all plants died in the extreme alkaline soil. It might be noted that the natural area where the very alkaline soil occurred was devoid of any vegetation.

Extraction of this plant proved un-feasible due to extremely low HC yield.

Figure 4. Entire Firecracker sunflower plant growing in very alkaline soil. The plant (~5 cm above ground) was harvested the same day at the 12-28 cm tall plants seen in Figure 3 (above).

Soil analyses of the alkaline soils from St. George, UT revealed the alkaline (locally called 'top soil') is similar to 'very alkaline' but the 'very alkaline' soil has about 4 times as much Calcium (Ca) and Sulfur (S), Both soils are generally low in K, Mg, Na, Nitrogen, Mn, Fe, Cu, and B (Fig. 5). It is easy to see that both soils, but especially the very alkaline soil, are difficult to obtain plant growth.

SAMPLE ID	LAB NUMBER	Organic Matter		Phosphorus		Potassium	Magnesium	Calcium	Sodium	
		* % Rating	** ENR lbs/A	P1 (Weak Bray) **** * ppm	$NaHCO_3$-P (OlsenMethod) **** * ppm	K ***** * ppm	Mg *** * ppm	Ca *** * ppm	Na *** * ppm	Soil pH
alkaline	54705	0.8L	46	1 *	13M	86L	216M	2602VH	49L	7.6
very alkaline	54706	2.4M	79	7 *	4L	206L	418L	10580VH	172L	7.8

SAMPLE NUMBER	Nitrogen NO_3-N ppm	Sulfur SO_4-S ppm	Zinc Zn ppm	Manganese Mn ppm	Iron Fe ppm	Copper Cu ppm	Boron B ppm	Excess Lime Rating	Soluble Salts mmhos/cm	Chloride Cl ppm
alkaline	2VL	792VH	1.2M	1VL	3VL	0.4L	0.9M	H	2.9H	
very alkaline	13M	10580VH	0.2VL	1VL	1VL	0.2VL	6.1VH	M	4.1VH	

Figure 5. Soil analyses of alkaline and very alkaline soils from St. George, UT. Only one plant survived in the 'very alkaline' soil (see Fig. 3). *Weak Bray unreliable at M or H excess lime or pH > 7.5.

Due to the unexpected CovID-19 virus pandemic that displaced the experiment from the PVHS greenhouse to the lab growth chamber, it is not statistically valid to compare the current results with the previous study on salt concentrations and sand (Johnson, Theobald and Adams 2019). However, it is worthwhile to make some qualitative estimates (Fig. 6). Munchkin plants grown in alkaline soil had much lower % HC yield (3.16), in contrast plants in high salt treatments that yielded large % HC yields. The plants growing in alkaline soil also had much lower biomass (comparable to the pure sand test), and much lower g HC/ g 10 leaves (again comparable to the sand treatment). It was thought (Johnson et al. 2020) that the sand was nutrient limiting (low in N, and organic matter) leading to low growth, and that is likely a factor for the alkaline soil.

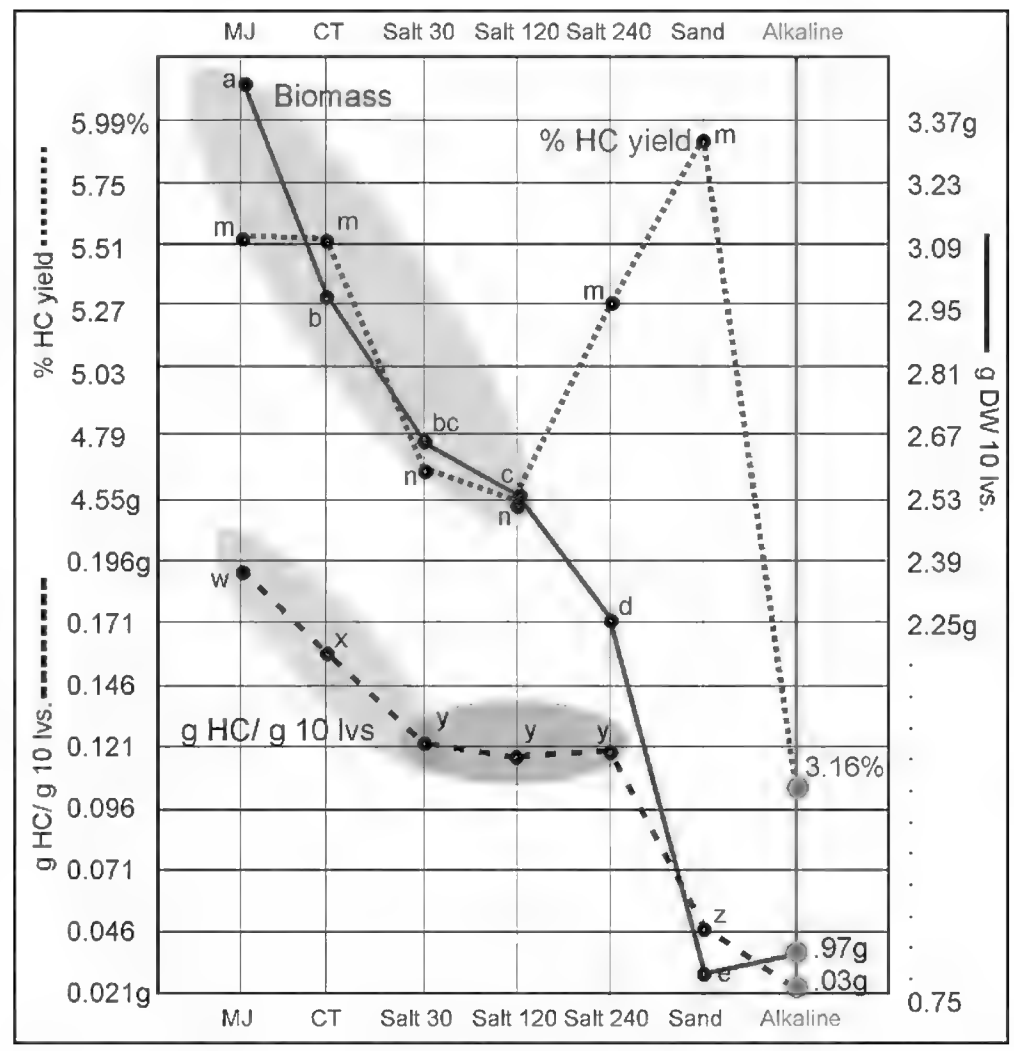

Fig. 6. Qualitative comparison of Munchkin's HC data with CT (Munchkin) in potting soil and especially in sand.

The three sunflower cultivars (Munchkin, Firecracker and Little Becka) varied in their ability to grow in alkaline, St. George soil. Munchkin was lowest in biomass, % HC yields, and g HC/ g DW 10 leaves. Little Becka was highest in % HC yields. Little Becka and Firecracker were (statistically) about equal in their biomass and g HC/ g DW 10 leaves yields. Overall, all the cultivars struggled to grow in the difficult alkaline soil with calcium levels of 2,602 ppm and sulfur 792 ppm, and being low in nitrogen and critical trace metals.

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Nuclear and chloroplast DNAs reveal diverse origins and mis-identifications of *Juniperus* cultivars from Windsor Gardens, UK, Part 3 of 3.

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ABSTRACT

Ploidy was determined for 15 plants labeled as *Juniperus squamata* at the Windsor Gardens, UK and revealed 12 were tetraploids (2*n*=4*x*=44) and 3 were diploids (2*n*=2*x*=22). nrDNA (ITS) and cp DNA sequencing the tetraploids found: 4 *J. squamata* (4x); 4 *J. tibetica* (4x) x *J. squamata* (4x); 2 *J. sabina* var. *balkanensis* (4x) x *J. squamata* (4x); and one *J. chinensis* var. *sargentii* (4x) x *J. squamata* (4x). Sequencing the 3 diploids revealed: 2 *J. pingii* (2x) x *J. pingii* (2x); and 1 J. *pingii* (2x)? x *J. komarovii*(2x)? Ploidy analyses of 18 additional cultivars, putatively from *Juniperus davurica, J. recurva, J. rushforthiana, J. sabina,* and *J. virginiana* revealed 6 diploids, 5 triploids and 7 tetraploids. Cultivar 'Musgrave' (4x), by DNA, was identical to *J. xpfitzeriana* 'Wilhelm Pfitzer' (4x). The DNA of the 5 triploids were all nearly identical to *J. xpfitzeriana* 'Wilhelm Pfitzer' (4x). 'Tamariscifolia' and 'Variegata' both had *J. sabina* var. *sabina* as their maternal parent, but the first had *J. sabina* var. *balkanensis* as the male parent and the second had *J. sabina* var. *sabina* as the male parent. Thus, 'Tamariscifolia' is the first discovery of a *J. sabina* var. *balkanensis* x *J. s.* var. *sabina* hybrid in cultivation. None of the 3 'davurica' cultivars proved to be *J. davurica*, but rather *J. chinensis* var. *procumbens* x *J. chinensis* var. *sargentii*. Cultivars *J. indica* and recurva 'densa' were shown to be *J. indica* var. *caespitosa.* recurva ' Embley Park' appears to be *J. coxii* x *J. squamata* var. *wilsonii*. *J. wallichiana* (=*J. indica*) 15460 was found to be *J. rushforthiana*, whereas *J. wallichiana* (15487) was discovered to be *J. indica* x *J. rushforthiana*. Cultivar *virginiana* 'cannaertii' was shown to be *J. virginiana*. Botanic gardens provide a great opportunity for species to hybridize with other species that are not in contact in nature. The species care and suitable habitat provided in a garden setting, as well as vegetative propagation methods have allowed the preservation of those rare hybrids). Identification of juniper hybrids and variants is quite imprecise. DNA barcoding of cultivated plants in botanic gardens would greatly facilitate the recognition, study and utilization of rare hybrids and somatic mutations.

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KEY WORDS: *Juniperus davurica, J. recurva, J. rushforthiana, J. sabina, J. squamata, J. virginiana,* cultivars, origin, nrDNA, ITS, cp DNA, DNA barcoding.

This is the third report in an on-going study (Adams et al. 2019, Adams et al. 2020) on DNA barcoding of *Juniperus* at Windsor Gardens. Our initial study of *Juniperus* x *pfitzeriana* cultivars at Windsor Gardens (Adams, et al. 2019) discovered all of the 14 cultivars were identical in their chloroplast DNA, which was identical to that of *J. sabina* var. *balkanensis* (Table 1). In addition, 13 *J.* x*pfitzeriana* cultivars were allo-tetraploids with heterozygous bases at 5 to 7 sites that distinguish *J. chinensis* and *J. sabina* var. *balkanensis*. These cultivars had identical nrDNA. Two cultivars, 'Old Gold' and 'Sea Green', showed a slightly different nrDNA pattern, being homozygous at sites 410 and 1139, as found in *J. s.* var. *balkanensis*. The origin of *J.* x*pfitzeriana* is from a cross of a male, tetraploid *J. sabina* var. *balkanensis* and a female, tetraploid, *J. chinensis*, resulting in an allo-tetraploid, dioecious, *J.* x*pfitzeriana* (Spath) Schmidt.

Table 1. nrDNA (ITS) variable sites in *J. chinensis* cultivars. (Windsor Gardens), *J. chinensis*, and *J. sabina*. K=G/T; S=C/G; Y=C/T; M=A/C; W=A/T; R=A/G. chloroplast types: *balkanensis* = *J. sabina* var. *balkanensis*/ *J. thurifera*; *sabina* = *J. sabina* var. *sabina*; and *chinensis* = *J. chinensis*. Modified from Adams et al. (2019). Site numbers modified to correspond with site numbers in Table 3 of this report.

taxa: *J.* x*pfitzeriana* (=x*media*) unless noted otherwise	ploidy	212[a] K	410 S	665 Y	986 Y	996 M	1034 K	1073 W	1137 R	ITS classification hybrid?	chloroplast, ex. pollen from:
Probable male (pollen) parent	4*x*	G	C	T	T	A	T	T	G	*J. sabina* var. *balkanensis*	*J. sabina* var. *balkanensis*
Probable female parent genotype	4*x*	T	G	C	C	C	G	A	A	*J. chinensis*	*J. chinensis*
15442 Arctic	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15454 Armstrongii	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15418 Aurea, Paris-sud	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15474 Aurea	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15423 Saybrook Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15425 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15463 Carberry Gold	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15443 Gold Star	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15462 Golden Saucer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15482 Goldenkissen	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15430 pfitzeriana prostate	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15435 Wilhelm Pfitzer	4*x*	G/T	C/G	C/T	C/T	A/C	G/T	A/T	A/G	chin x sab	*balkanensis*
15453 Old Gold	4*x*	G/T	**C**	C/T	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15436 Sea Green, Windsor	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis*
15604 Sea Green Home Depot	3x	G/T	**C**	**T**	C/T	A/C	G/T	A/T	**G**	chin x sab*	*balkanensis?*

[a]Variable sites located at: 212, xGGCCAAGC; 410, xGTTGAGAT; 665, xTCTTCGTC; 986, xGCCCTCCC; 996, xGCGAGGAG; 1034, xGCGGTCGG; 1073, xCGCGACGA; 1137, xGAACTTTG.

In our second study of 24 *J. chinensis* cultivars at Windsor Gardens, we reported (Adams et al. 2020, this issue) that two cultivars were found to be mis-identified, and were actually *Cupressus gigantea* and *J. virginiana*. Interestingly, of the remaining 22 '*chinensis* cultivars' only 3 plants were 'pure, autotetraploid '*J. chinensis*' by DNA sequencing and Flow Cytometry (FC) ploidy determination. The parentage of the remaining 19 samples had mixed parents from several related species.

The purpose of the present research is to present new DNA sequencing utilizing both chloroplast and nuclear DNA to determine variation in *Juniperus davurica, J. recurva, J. rushforthiana, J. sabina, J. squamata,* and *J. virginiana,* cultivars at Windsor Gardens.

METHODS

Plant materials:
Samples: Leaf samples were collected in Windsor Gardens, Windsor Great Park, Windsor, *SL4 2HT* UK from 33 *Juniperus* cultivar accessions (see Table 2) and immediately placed in activated silica gel for DNA sequencing and Flow Cytometry - ploidy determination (Table 2).

Table 2. Windsor 33 *Juniperus* cultivars collected with cultivar origin (< is earlier than).

taxon (as labeled at Windsor Garden)	Adams. Coll. #	Windsor acc. #	ploidy (this study)	Chrom. number, *2n*, litr.	Origin: based on Den Oden and Boom 1965; Krussmann 1991; Welch 2012, Lewis 1998, Auders & Spicer 2012
davurica 'Expansa'	*15431*	2001-448	4x		Netherlands 1940
davurica 'Expansa Variegata'	*15475*	1999-5915	4x		Netherlands 1938
davurica 'Expansa Aureo-spicata'	*15444*	1999-5914	4x		Netherlands 1940
indica'	*15437*	1999-6150	2x		unknown
pingii var. wilsonii	*15459*	2000-1308	2x		China 1910
recurva 'densa'	*15419*	1999-5967	2x	44	UK 1862
recurva 'Embley Park'	*15420*	1999-2968	2x	44	UK 1961
sabina 'Variegata'	*15434*	2001-405	2x		UK 1822
sabina 'Musgrave'	*15479*	1999-5986	4x		UK 1930
sabina 'Tamariscifolia'	*15489*	1999-5991	4x	22,44	UK 1789
squamata	*15483*	1999-6162	4x	44	unknown
squamata	*15456*	1999-6161	4x	44?	unknown
squamata	*15485*	1999-6163	2x	44	unknown
squamata 'Blue Alps'	*15447*	1999-6022	4x	44?	UK/Austria 1968
squamata 'Blue Spider'	*15481*	1999-6024	4x	44?	Netherlands <1980
squamata 'Chinese Silver'	*15455*	1999-6027	4x	44?	UK 1964. TTYu 7881/TTYu 15614
squamata 'Filborna'	*15476*	1999-6028	4x	44?	Sweden 1946
squamata 'Glassell'	*15445*	1999-6029	4x	44?	UK 1958
squamata 'Holger'	*15486*	1999-6031	4x	44?	Sweden 1946
squamata 'Prostrata'	*15421*	1999-6036	2x	(44)	UK ?
squamata 'Pygmaea'	*15424*	2001-778	2x	(44)	UK <1964
squamata var. fargesii	*15480*	1999-6167	4x	44?	China Rehder & Wilson 1914
squamata 'Wilsonii'	*15449*	1999-4563	4x	44?	China 1910
squamata 'Wilsonii'	*15450*	1999-6038	4x	44?	China 1910
squamata 'Yellow Tip'	*15457*	1999-6039	4x	44?	Netherlands <1991
virginiana Pfitzer Group 'Hetzii'	*15422*	2000-521	**3x**	33	USA 1920
virginiana 'Glauca' x Pfitzer Group) '= Grey Owl'	*15429*	2000-266	**3x**	33	Netherlands 1938
virginiana Pfitzer Group 'Sulphur Spray'	*15438*	1999-6114	**3x**	33	Netherlands 1962
virginiana 'cannaertii'	*15440*	1999-6045	2x	22	Belgium 1868
virginiana 'Glauca' x Pfitzer Group) = 'Grey Owl'	*15448*	1999-6149	**3x**	33	Netherlands 1938
virginiana 'Blue Cloud'	*15468*	1999-6042	**3x**	33	Netherlands 1955
wallichiana	*15460*	2000-571	4x		unknown
wallichiana	*15487*	1999-6144	4x		unknown

DNA extraction and sequencing

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Chromas 2.31 (Technelysium Pty Ltd.) was used viewing sequence chromatograms and Mafft used for alignment.

Flow cytometric analyses for ploidy level determination

Nuclear DNA amount was assessed by flow cytometry (FC) based on the technique of Bourge et al. (2018) on silica dried leaves of *Juniperus* samples and fresh leaves of *Hordeum vulgare* L. 'Sultan' [2C= 9.81 pg in Garnatje et al. (2004)] used as an internal standard. Approximately, 30 mg of leaves of both the internal standard and *Juniperus* were simultaneously chopped using a razor blade in a plastic Petri dish with 500 µl of cold Gif nuclear-isolation buffer-GNB (Bourge et al. 2018): 30 mM sodium citrate, 45 mM $MgCl_2$, 60 mM MOPS (4-morpholine propane sulphonate, pH 7), and 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2 containing 0.1% (w/v) Triton X–100, supplemented with 10 mM sodium metabisulphite and RNase (2.5 U/ml). The nuclei suspension was filtered through 50 µm nylon mesh. The nuclei were stained with 100 µg/ml propidium iodide (PI), a specific DNA fluorochrome intercalating dye, and kept at 4°C for 5 min. DNA content of about 3,000 stained nuclei was determined for each sample using the cytometer CytoFLEX S (Beckman Coulter- Life Science United States. Excitation 561 nm, 26 mW; emission through a 620/10 nm band-pass filter). Measurements of each sample were repeated twice. The software CytExpert was used for histogram analyses. The total 2C DNA value was calculated using the linear relationship between the fluorescent signals from stained nuclei of the species and the internal standard, according to the following formula:

2C DNA sample (pg) = (Sample 2C peak mean / Standard 2C peak mean) x Standard 2C DNA (pg).

RESULTS AND DISCUSSION

Analyses of *Juniperus 'squamata'* cultivars (15)

Ploidy levels determined for 15 *Juniperus 'squamata'* cultivars revealed that 12 are tetraploids (4x) and 3 are diploids (2x, Table 3). It should be noted that all the *'squamata'* cultivars were shrubs, not trees.

Analyses of trnSG (SG hereafter) found the paternal (male, pollen) parent varied considerably with 4 cultivars having SG cp DNA about equally similar to *J. pingii* or *J. squamata* (green, Table 3). *Juniperus tibetica* (or a shrub form in cultivation) appears to be the paternal parent of 5 cultivars, 15450 'Wilsonii', 15479 'Filborna', 15480 'var. fargesii', 15481 'Blue Spider', and 15449 'Wilsonii' (Table 3). Two cultivars, 'Yellow Tip' and 'Holger', have the cp of *J. thurifera* or *J. sabina* var. *balkanensis*. Because *J. thurifera* is a tree and *J. s.* var. *balkanensis* is a shrub, it seems more likely the paternal parent is *J. s.* var. *balkanensis*. 'Blue Alps' is interesting as its male parent is *J. chinensis* var. *sargentii* (Table 3).

nrDNA (ITS) indicates only 4 cultivars appear to be from *J. squamata* x *J. squamata* parents (in green, Table 3). Most of the 15 cultivars have a maternal parent of *J. squamata* or a closely related taxon (Table 3). The nrDNA for *15450 J. squamata* 'Wilsonii' was equal to *J. pingii* and *J.* squamata, but as *J. pingii* a diploid, that favors *J. squamata* as the maternal parent. Both *15450 J. squamata* 'Wilsonii' and

15450 J. squamata 'Wilsonii' appear to be of hybrid origin (*J. tibetica* x *J. squamata*), but as *J. tibetica* is a tree (in the wild), there may be a shrub form in cultivation that is the male parent. The parents of 15480 'var. fargesii' and 15481 'Blue Spider' appear to be *J. tibetica* (or a shrub form in cultivation) x *J. squamata.* Plant 15449 'Wilsonii', has ITS DNA most similar to *J. squamata* var. *wilsonii*, but not definitive. It is likely that its ITS sequences are not yet in GenBank.

Both 'Yellow Tip' and 'Holger' seem derived from a *J. sabina* var. *balkanensis* x *J. squamata* cross. This is of some interest because *J. s.* var. *balkanensis* and *J. squamata* do not grow near each other in nature (Adams 2014), so the *J. s.* var. *balkanensis* male parent must be (or have been) in cultivation in a garden where the cross occurred. So far as known, var. *balkanensis* has not been found in cultivation. The origin of 'Holger' (Sweden, 1946) is older than that of 'Yellow Tip' (Netherlands, 1991, Table 1), so it is possible that 'Holger' was acquired later in the Netherlands (1991) and a yellow, somatic mutation occurred, thence the new cultivar 'Yellow Tip'.

Two of the diploids *15421* 'Prostrata' (UK ?) and *15424 J. squamata* 'Pygmaea' (UK before 1964) have identical male and female parent matches (Table 3) and their ITS differs by only 1 bp. The third diploid, *15485 squamata* (origin unknown) differs by 3 bp in ITS and has quite different putative parents (pingii x komarovii). As both *J. pingii* and *J. komarovii* are trees, this seems unlikely.

Table 3. ITS (13 informative ITS SNPs) and trnS-trnG (cp) analyses of *J. squamata* cultivars at Windsor Gardens.

accession name at Windsor Gardens, with ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et al. 2019	maternal parent based on ITS, with ploidy from Farhat et al. 2019	putative origin of Windsor Gardens accession, with ploidy (this study) (paternal x maternal)
15445 J. squamata 'Glassell' 4x	pingii 2x/ squamata 4x	squamata? 4x	squamata x squamata 4x
15455 J. squamata 'Chinese Silver' 4x	pingii 2x/ squamata 4x	squamata 4x	squamata x squamata 4x
15483 J. squamata 4x	pingii 2x/ squamata 4x	squamata? 4x	squamata x squamata 4x
15456 J. squamata 4x	pingii 2x/ squamata 4x	indica 4x x squamata 4x?	squamata x squamata 4x
15480 J. squamata var. fargesii 4x	tibetica 4x	tibetica 4x/squamata 4x	tibetica x squamata 4x
15481 J. squamata 'Blue Spider' 4x	tibetica 4x	tibetica 4x/ squamata 4x	tibetica x squamata 4x
15449 J. squamata 'Wilsonii' 4x	tibetica 4x	wilsonii 4x/ squamata 4x	tibetica x sq. var. wilsonii? 4x
15450 J. squamata 'Wilsonii' 4x	tibetica 4x	pingii 2x/ squamata 4x	tibetica x squamata 4x
15476 J. squamata 'Filborna' 4x	tibetica 4x	squamata 4x	tibetica x squamata 4x
15457 J. squamata 'Yellow Tip' 4x	thurifera 4x /sabina var. balkanensis 4x	squamata 4x	sabina var. balkanensis x squamata 4x
15486 J. squamata 'Holger' 4x	thurifera 4x /sabina var. balkanensis 4x	squamata 4x	sabina var. balkanensis x squamata 4x
15447 J. squamata 'Blue Alps'	sargentii	squamata 4x	sargentii x squamata
15421 J. squamata 'Prostrata' 2x	pingii 2x/ squamata 4x	pingii 2x	pingii/squamata x pingii 2x
15424 J. squamata 'Pygmaea' 2x	pingii 2x/ squamata 4x	pingii 2x	pingii/squamata x pingii 2x
15485 J. squamata 2x	pingii 2x/ squamata 4x	komarovii 2x	pingii? x komarovii? 2x

Four of the 'squamata' cultivars were clearly hybrids in their nrDNA (Table 4) with 13 heterozygous sites in 957 bp sequenced and a region with slipped sequences (427-750) with flanking indels that prevented sequencing. No differences were found among the 4 hybrids (in the 957 bp sequence), except at sites 802 and 995 (Table 4), yet, their putative male parents were all *squamata* (Table 3).

Table 4. Thirteen (13) heterozygous nrDNA sites in 4 hybrids, identical, except at sites 802 and 995.

hybrids	site# 179	212	351	363	365	366	389	no seq 427-750	802	985	995	1071	1243	1169
15476 Filborna 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A/G	C/T	A/C	A/T	A/G	C/T
15457 Yellow Tip 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A/G	C/T	A/C	A/T	A/G	C/T
15486 Holger 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	A	C/T	C	A/T	A/G	C/T
15447 Blue Alps 4x	C/T	G/T	C/T	C/G	C/G	C/T	C/G	na	G	C/T	C	A/T	A/G	C/T

Analyses of the other 18 *Juniperus davurica, J. recurva, J. rushforthiana, J. sabina, J. virginiana* cultivars

This group contained 6 diploids, 5 triploids, and 7 tetraploids (Table 5). The nrDNA of 15479 sabina 'Musgrave' (4x) was found to be identical to 15435 xpfitzeriana 'Wilhelm Pfitzer', considered the 'mother' of all pfitzer cultivars (Adams et al. 2019). All of the triploids were similar or somewhat similar to 'xpfitzeriana' in their ITS (Table 5). Both 15489 sabina 'Tamariscifolia' (4x) and 15434 sabina 'Variegata' (2x) had identical ITS DNA, which was 100% identical to *Adams 14317*, *J. sabina*, Type 2 ITS from Azerbaijan. This is suggestive that 'Tamariscifolia' might be an auto-tetraploid from 'Variegata'.

All three davurica cultivars were tetraploids that had NCBI matches of 99.31 to 99.74% to *J. chinensis* var. *sargentii* (no origin listed in NCBI). No heterozygous sites were found suggesting the tetraploid cultivars are auto-tetraploids. Two cultivars, 15437 indica and 15437 recurva 'densa', both diploids, had ITS sequences nearly identical (99.82, 99.85%) to *J. indica* var. *caespitosa*, a shrub, in contrast to *J. indica* (var. *indica*), a tree.

Accessions 15420 recurva 'Embley Park' 15459 'pingii var. wilsonii' had 100% matches to *J. squamata* var. *wilsonii* (*Adams 5521*, Arnold Arboretum).

Both accessions named 'wallichiana' (treated as *J. indica* in Adams 2014), were 99.83 and 99.49% similar to *Adams 8140*, ex Bhutan (from a field collection by K. Rushforth). 15460 differed at site 167, being heterozygous (Table 5). Finally, 15440 virginiana 'cannaertii' (2x) had a 99.37% match to *J. virginiana*, *Adams 10231*, Knoxville, TN

Analysis of trnSG (cp DNA) confirmed the paternal parent (by pollen) of 15479 sabina 'Musgrave' was *J. sabina* var. *balkanensis*, being the same as found in the Wilhelm Pfitzer (Table 6). All of the triploids plus the tetraploid 'Tamariscifolia' were also found to have *J. sabina* var. *balkanensis* as the paternal parent.

Surprisingly, 15434 sabina 'Variegata', which had identical ITS DNA with 'Tamariscifolia' (Table 5), had cp DNA of *J. sabina* var. *sabina* (100% to *Adams 14317*, Azerbaijan). In contrast, Tamariscifolia had *J. sabina* var. *balkanensis* cp DNA.

Table 5. ITS classification of the 18 cultivars. 15435 xpfitzeriana 'Wilhelm Pfitzer' from Adams et al. (2019) is included as a pfitzer exemplar. MAFFT and NCBI (BLASTn) search reported at % pairwise similarity (i.e. 100% = identical sequences, etc.)

Adams coll. #, Windsor accession name, ploidy(this study)	ITS classification, ploidy from Farhat et al. 2019.	168 C/T	212 G/T	350 A/G	410 C/G	663 C/T	985 C/T	995 A/C	1033 G/T	1071 A/T	1135 A/G	1147 A/T
15435 xpfitzeriana 'Wilhelm Pfitzer ex Adams et al. 2019.	**J. xpfitzeriana (hybrid sabina v. balkanensis 4x X chinensis [1]**	**C**	**G/T**	**A**	**C/G**	**C/T**	**C/T**	**A/C**	**G/T**	**A/T**	**A/G**	**T**
15479 sabina 'Musgrave'	J. xpfitzeriana	C	G/T	A	C/G	C/T	C/T	A/C	G/T	A/T	A/G	T
15438 virginiana Pfitzer Group 'Sulphur Spray' 3x	~= J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x[1]	C/T	G/T	A/G	C/G	C/T	C/T	A/C	G/T	A/T	A/G	A/T
15422 virginiana Pfitzer Group 'Hetzii' 3x	~= J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x[1]	C/T	G/T	A/G	C/G	T	C/T	A/C	G	A/T	A/G	A/T
15429 virginiana 'Glauca' = 'Grey Owl' 3x	~= J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x[1]	C/T	G/T	A/G	C/G	T	C/T	A/C	G	A/T	A/G	A/T
15448 virginiana 'Glauca' = 'Grey Owl' 3x	~= J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x[1]	C/T	G/T	A/G	C	T	C/T	A/C	G	A/T	G	A/T
15468 virginiana 'Blue Cloud' 3x	~= J. xpfitzeriana 4x sabina v. balkanensis 4x X chinensis 4x[1]	C/T	G/T	A/G	C	T	C/T	A/C	G	A/T	G	A/T
15489 sabina 'Tamariscifolia' 4x	J. sabina var. sabina, Type 2, ITS 2x	MAFFT 100%, to *Adams 14317*, Azerbaijan, Type 2 ITS **Note** Identical to 15434 Variegata										
15434 sabina 'Variegata' 2x	J. sabina var. sabina, Type 2, ITS 2x	MAFFT 100%, to *Adams 14317*, Azerbaijan, Type 2 ITS **Note** Identical to 15489 Tamariscifolia										
15444 davurica 'Expansa Aureo-spicata 4x	J. chin. var. sargentii 4x	NCBI 99.31%, origin of J. c. var. sargentii, not listed in NCBI										
15475 davurica 'Expansa Variegata' 4x	J. chin. var. sargentii 4x	NCBI 99.74%, origin of J. c. var. sargentii, not listed in NCBI										
15431 davurica 'Expansa' 4x	J. chin. var. sargentii 4x	NCBI 99.74%, origin of J. c. var. sargentii, not listed in NCBI										
15437 indica 2x	J. indica v. caespitosa 2x?	NCBI 99.82% to *Adams 7625*, Nepal										
15419 recurva 'densa' 2x	J. indica v. caespitosa 2x?	NCBI 99.85% to *Adams 7625*, Nepal										
15420 recurva 'Embley Park' 2x	J. squamata var. wilsonii 2x	MAFFT 100% to Adams, 5521, Arnold Arbor., #1010-64A										
15459 pingii var. wilsonii 2x	J. squamata var. wilsonii 2x	MAFFT 100% to Adams, 5521, Arnold Arbor., #1010-64A										
15460 wallichiana 4x	J. rushforthiana 4x	NCBI 99.83% to *Adams 8140*, Bhutan, site 167 A/G										
15487 wallichiana 4x	J. rushforthiana 4x	NCBI 99.49% to *Adams 8140,* Bhutan										
15440 virginiana 'cannaertii' 2x	J. virginiana 2x	NCBI 99.37% to *Adams 10231*, Knoxville, TN										

All three 'davurica' accessions had *J. chinensis* var. *procumbens* (4x) as the paternal parent. The diploid accessions, 15437 indica, and 15419 recurva 'densa' had *J. indica* var. *caespitosa* as the paternal parent (Table 6).

Accession 15419 recurva 'densa' had a 100% match to *J. coxii* in GenBank (origin not given), but as *J. coxii* is a tetraploid, this should be viewed some caution. *15459* pingii var. wilsonii (2x), had matches of 99.92% to *J. pingii* (*Adams 8506,* tree, Yunnan) and *J. carinata* (*Adams 8498,* shrub, Yunnan), because *J. carinata* is a shrub (as is acc. 15459), this favors *J. carinata* as the pollen parent.

Interestingly the 2 'wallichiana' tetraploids had different paternal parents: *J. rushforthiana* (4x) for 15460, and *J. indica* (2x), for 15487 (Table 6). 15440, virginiana 'cannaertii' (2x), had the cp of *J. virginiana* (2x).

Table 6. Analyses of putative paternal (pollen) parents by trnSG cp DNA.

Adams coll #, accession name at Windsor Gardens, and ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et. al. 2019	notes on identification
15435 xpfitzeriana 'Wilhelm Pfitzer'	J. sabina var. balkanensis (Adams et al. 2019)	J. xpfitzeriana (hybrid sabina v. balkanensis 4x X chinensis 4x (Adams et al. 2019)
15479 sabina 'Musgrave'	J. sabina var. balkanensis	MAFFT 99.92%, *Adams 13725*, Bulgaria
15438 virginiana Pfitzer Group 'Sulphur Spray' 3x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15422 virginiana Pfitzer Group 'Hetzii' 3x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15429 virginiana 'Glauca' = 'Grey Owl' 3x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15448 virginiana 'Glauca' = 'Grey Owl' 3x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15468 virginiana 'Blue Cloud' 3x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15489 sabina 'Tamariscifolia' 4x	J. sabina var. balkanensis 4x	MAFFT 99.92%, *Adams 13725*, Bulgaria
15434 sabina 'Variegata' 2x	J sabina var. sabina 2x	MAFFT 100.0%, *Adams 14317*, Azerbaijan
15444 davurica 'Expansa Aureo-spicata 4x	J. chinensis var. procumbens 4x	NCBI 100.0%, no origin given
15475 davurica 'Expansa Variegata' 4x	J. chinensis var. procumbens 4x	NCBI 100.0%, no origin given
15431 davurica 'Expansa' 4x	J. chinensis var. procumbens 4x	NCBI 100.0%, no origin given
15437 indica 2x	J. indica var. caespitosa 2x?	MAFFT 99.92%, *Adams 7625*, Nepal
15419 recurva 'densa' 2x	J. indica var. caespitosa 2x?	NCBI 99.85%, *Adams 7625*, Nepal
15420 recurva 'Embley Park' 2x	J. coxii 4x	NCBI 100.0%, no origin given
15459 pingii var. wilsonii 2x	J. pingii (tree) 2x/ J. carinata (shrub) 2x	MAFFT 99.92%, *Adams 8506*, China MAFFT 99.92%, *Adams 8498*, China
15460 wallichiana 4x	J. rushforthiana 4x	MAFFT 100.0%, *Adams 8140*, Bhutan
15487 wallichiana 4x	J. indica 2x	NCBI 99.85%, no origin given
15440 virginiana 'cannaertii' 2x	J. virginiana 2x	MAFFT 100.0%, *Adams 10231* TN, USA

A summary of the paternal and maternal parents and putative origin of these 18 cultivars is given in Table 7. Notice that xpfitzeriana 'Wilhelm Pfitzer', 'Musgrave' and all the triploids have the same parents: male, *J. sabina* var. *balkanensis*; female, *J. chinensis* (Table 7). However, the triploids, seem unlikely to have come from 2 tetraploid parents. Farhat et al. (2019) reported only tetraploids in *J. chinensis* accessions, but cited literature reports of *J. chinensis* diploids. So, perhaps a diploid *J. chinensis* is the maternal parent of all the triploids. Alternatively, perhaps a meiotic abnormality occurred in in *J. sabina* var. *balkanensis*, producing haploid pollen that led to the first triploid in this group. Then, subsequent selection for somatic mutation(s) in a vegetative character led to the cloning of that (those) 'sports', and thence to the other triploid cultivars. They are certainly very closely related in their DNAs and appear to be as similar as siblings.

Cultivar 15434 sabina 'Tamariscifolia' is interesting in that it has *J. sabina* var. *balkanensis* (4x) as the paternal parent, but *J. sabina* var. *sabina* Type 2 ITS (2x) as the maternal parent. ITS Types 1 and 2 DNAs differ by 8 sites (Adams et al. 2018a,b), and both var. *balkanensis* and var. *sabina* have Types 1 and 2 ITS DNA, as well as numerous occurrences of hybridization between Type 1 and Type 2 plants (heterozygous for some or all of the 8 sites, Adams et al. 2018a,b). The origin of 'Tamariscifolia' seems to be from a reduced male gamete (2x pollen) of var. *balkanensis* fertilizing a diploid (un-reduced gamete, 2x) of var. *sabina* (Table 7). It is interesting that Le Duc et al. (1999) found that 'Tamariscifolia' grouped with *J. sabina* in PCO ordination using RAPDs (Random Amplified Polymorphic DNAs).

Complementing the origin of 'Tamariscifolia' is the origin of 'Variegata', a diploid arising from pollen of *J. sabina* var. *sabina* (2x, Type 2 ITS), fertilizing *J. sabina* var. *sabina* (2x, Type 2 ITS). Type 2 ITS for both parents was deduced by the lack of heterozygous sites in the ITS DNA for accession 'Variegata'.

None of the Windsor 'davurica cultivars' were, in fact, related to *J. davurica*. Juniperus davurica grows in Mongolia and far eastern Russia. It seems unlikely that it is in cultivation in nurseries and Botanic gardens. All 3 of these 'davurica cultivars' have *J. chinensis* var. *procumbens* as the pollen (paternal) parent and *J. chinensis* var. *sargentii* as the maternal parent (Table 7) and both parents are tetraploids as well as cultivars Expansa Aureo-spicata and Expansa Variegata. However, Windsor davurica 'Expansa' was found to be a triploid, indicating that one of the parents might be a diploid *J. chinensis* taxon.

The parents of 15437 indica (2x) and 15419 recurva 'densa' appear to both be *J. indica* var. *caespitosa* (2x), the shrubby variety of *J. indica* var. *indica*, a tree. The diploid, recurva 'Embley Park' (15420) had cp of *J. coxii* (or perhaps a close relative that is 2x) and *J. squamata* (4x) (or a relative that is diploid) as parents (Table 7).

A pingii plant seems unusual in cultivation as *J. pingii* is a large tree in Kunming, China. It appears that 15459 *pingii* var. *wilsonii* (2x) male parent is likely *J. carinata* (a shrub, 2x) rather than *J. pingii* (a tree). The maternal parent is *J. squamata* var. *wilsonii* (2x) or a relative that is diploid (Table 7). The 15460 'wallichiana' cultivar seems to be a good *J. rushforthiana*. But 15487 'wallichiana' appears to have a *J. indica* pollen parent and *J. rushforthiana* maternal parent. Finally, 15440 virginiana 'cannaertii' (2x), is *J. virginiana* by both parents.

Table 7. Putative origin of 18 cultivars at Windsor Gardens.

Adams coll. #, acc. name at Windsor Gardens, and ploidy (this study)	paternal (male) parent by trnSG cp sequence, with ploidy from Farhat et al. 2019	maternal parent based on ITS, with ploidy from Farhat et. al. 2019	putative origin of Windsor Gardens accession, with ploidy (this study) (paternal x maternal)
15435 xpfitzeriana 'Wilhelm Pfitzer'	J. sabina var. balkanensis (Adams et al. 2019)	J. chinensis	J. xpfitzeriana; ie., J. sabina var. balkanensis x chinensis [1]
15479 sabina 'Musgrave'	J. sabina var. balkanensis	J. chinensis	J. xpfitzeriana; ie., J. sabina var. balkanensis x J. chinensis
15438 virginiana Pfitzer Group 'Sulphur Spray' 3x	J. sabina var. balkanensis 4x	J. chinensis 4x	~= J. xpfitzeriana; J. sabina var. balkanensis x J. chinensis 3x
15422 virginiana Pfitzer Group 'Hetzii' 3x	J. sabina var. balkanensis 4x	J. chinensis 4x	~= J. xpfitzeriana; J. sabina var. balkanensis x J. chinensis 3x
15429 virginiana 'Glauca' = 'Grey Owl' 3x	J. sabina var. balkanensis 4x	J. chinensis 4x	~= J. xpfitzeriana; J. sabina var. balkanensis x J. chinensis 3x
15448 virginiana 'Glauca' = 'Grey Owl' 3x	J. sabina var. balkanensis 4x	J. chinensis 4x	~= J. xpfitzeriana; J. sabina var. balkanensis x J. chinensis 3x
15468 virginiana 'Blue Cloud' 3x	J. sabina var. balkanensis 4x	J. chinensis 4x	~= J. xpfitzeriana; J. sabina var. balkanensis x J. chinensis 4x
15489 sabina 'Tamariscifolia' 4x	J. sabina var. balkanensis 4x	J. sab. var. sabina Type 2 ITS 2x	J. sabina v. balkanensis Type 2 ITS x J. sab var. sabina Type 2 ITS 4x
15434 sabina 'Variegata' 2x	J sabina var. sabina 2x	J. sab. var. sabina Type 2 ITS 2x	J. sab. var. sabina Type 2 ITS x J. sab. var. sabina Type 2 ITS 2x
15444 davurica 'Expansa Aureo-spicata 4x	J. chinensis var. procumbens 4x	J. chinensis var. sargentii 4x	J. chinensis var. procumbens x J. chinensis var. sargentii 4x
15475 davurica 'Expansa Variegata' 4x	J. chinensis var. procumbens 4x	J. chinensis var. sargentii 4x	J. chinensis var. procumbens x J. chinensis var. sargentii 4x
15431 davurica 'Expansa' 4x	J. chinensis var. procumbens 4x	J. chinensis var. sargentii 4x	J. chinensis var. procumbens x J. chinensis var. sargentii 4x
15437 indica 2x	J. indica var. caespitosa 2n?	J. indica var. caespitosa 2n?	J. indica var. caespitosa 2x
15419 recurva 'densa' 2x	J. indica var. caespitosa 2n?	J. indica var. caespitosa 2n?	J. indica var. caespitosa 2x
15420 recurva 'Embley Park' 2x	J. coxii 4x	J. squamata var. wilsonii 2x	J. coxii x J. squamata var. wilsonii 2x
15459 pingii var. wilsonii 2x	J. pingii (tree) 2x/ J. carinata (shrub) 2x	J. squamata var. wilsonii 2x	J. carinata x J. squamata var. wilsonii 2x
15460 wallichiana 4x	J. rushforthiana 4x	J. rushforthiana 4x	J. rushforthiana 4x
15487 wallichiana 4x	J. indica 2x	J. rushforthiana 4x	J. indica x J. rushforthiana 4x
15440 virginiana 'cannaertii' 2x	J. virginiana 2x	J. virginiana 2x	J. virginiana 2x

In this study, we found tremendous variation in the origin of cultivars as evidenced by highly diverse nrDNA and cp parentage. Botanic gardens provide unusual laboratories for the production of hybrids whose parents are seldom if ever sympatric in nature. *Juniperus* species from very diverse regions and habitats are grown in close proximity, under favorable conditions, such that opportunities for cross pollination are favorable. A hybrid seedling that grows under the maternal plant may be protected from weeding, and later discovered and rescued into a greenhouse. Survival in Botanic Gardens and private estates is common and has led to commercialization of many 'sports' (mutations) and hybrids that are now sold as cultivars. One has only to peruse books on cultivated conifers (Den Oden and Boom 1965; Krussmann 1991; Welch 2012) to see the number of bizarre shapes that have been cloned and propagated in the past two centuries. So, it is not surprising that this study revealed great variation in parentage and ploidy of the cultivars. The development and implementation of a DNA barcode system would greatly aid botanic gardens to screen current and incoming accessions to assign taxonomic names to junipers and other conifers.

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Table 3. ITS (13 informative ITS SNPs) and petN-psbM (cp), trnS-trnG (cp) analyses of *J. squamata* cultivars at Windsor Gardens.

accession name at Windsor Gardens	Adams coll. #	ploidy this acc.	Paternal ID by trnSG cp data	Windsor acc. identity by ITS data.	179[1] C/T	212 G/T	351 C/T	363 C/G	365 C/G	366 C/T	389 C/G	802 A/G	985 C/T	995 A/C	1071 A/T	1143 A/G	1169 C/T
J. squamata 'Blue Alps'	*15447*	4x	sargentii	sargentii x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	C	A/T	A/G	C/T
J. squamata 'Yellow Tip'	*15457*	4x	thurifera	thurifera x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
J. squamata 'Holger'	*15486*	4x	thurifera	thurifera x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
J. squamata 'Filborna'	*15476*	4x	tibetica	tibetica x squamata	C/T	G/T	C/T	C/G	C/G	C/T	C/G	A/G	C/T	A/C	A/T	A/G	C/T
ITS complement of 15480, 15481, etc. below	na	na	na	sargentii (NCBI blast)	C	T	T	C	G	C	C	G	C	A	A	G	T
J. squamata var. fargesii	*15480*	4x	tibetica	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Blue Spider'	*15481*	4x	tibetica	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Glassell'	*15445*	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Chinese Silver'	*15455*	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata	*15483*	4x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Wilsonii'	*15449*	4x	tibetica	wilsonii	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Wilsonii'	*15450*	4x	tibetica	pingii?	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata	*15456*	4x	pingii/squamata	indica/squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Prostrata'	*15421*	2x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata 'Pygmaea'	*15424*	2x	pingii/squamata	pingii/ squamata	T	G	C	G	C	T	G	A	T	C	T	A	C
J. squamata	*15485*	2x	pingii/squamata	pingii/squamata / komarovii	T	G	C	G	C	T	G	A	T	C	T	A	C

[1] 179-xGCGGACAC, 212- zGCCCAAGC, 351- xGTCGGAGC, 363,365,366- GAGCGAGxGyz, 389- xGAGGTCCG, 803-xAAACATAA, 985(492)-xGCCCTCCC, 995(502)-xGCGAGGAG, 1071(578)-xCGCGACGA, 1143(650)- xTCTTTGGT, 1169(676)- xGCGGGCAT.

Do Gas Exchange Rates of *Phaseolus texensis* (Boerne Bean, Leguminosae) Reflect its Potential Niche?

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ABSTRACT

Gas exchange rates were measured to examine potential niche requirements of *Phaseolus texensis* Delgato-Salinas and Carr (Boerne Bean, Leguminosae = Fabaceae) a rare species found in the Edwards Plateau physiographic region of Central Texas. Plants were found below a *Juniperus ashei/ Quercus fusiformis* (Ashe juniper/live oak) canopy at a light level of 169 ± 28 µmol/m^2/s (mean ± se). Light response curves were generated using photosynthetic rates of leaflets from plants below the tree canopy in shade. Gas exchange rates were measured at light levels from 0-2000 µmol/m^2/s. A number of photosynthetic parameters were calculated and then compared. The maximum photosynthetic rate (A_{max}) was 5.99 ± 0.17 µmol CO_2/m^2/s for leaflets of sub-canopy plants. Light saturation was 158 ± 42 µmol/m^2/s. Light levels below the canopy where plants were found was 7% higher than the light saturation point of *P. texensis*, which means the plants were fixing carbon at their highest level even in the low light, shaded, sub-canopy environment. At A_{max}, the light compensation point was 0.102 ± 0.099 µmol/m^2/s for sub-canopy plants and dark respiration was 0.235 ± 0.227 µmol/m^2/s. *Phaseolus texensis* exhibits photosynthetic characteristics equivalent to other shade plants which seem to be part of the reason it can persist in the understory of relatively open, but low light understory environments in isolated Central Texas woodland communities. *Published on-line* www.phytologia.org *Phytologia 102(3): 162-171 (Sep 21, 2020). ISSN 030319430.*

KEY WORDS: light levels; CO_2 uptake; photosynthetic rates; shade plants; sun plants; edge plants; light saturation; light compensation; respiration.

Understanding environmental factors controlling growth and recruitment of herbaceous and woody species in woodland communities is challenging, even though many papers have examined the topic (see Baker et al. 2005). This is especially true for populations of relic, endemic, threatened, and rare species (Falk et al. 1996; Poole et al 2007; Nelson Dickerson and Van Auken 2016).There are approximately 5,500 species of plants known in Texas (Correll and Johnston 1970) with 314 endemic or only occurring in Texas (Carr 2019). Of these, 75 or 23.5% occur in the central Texas Edwards Plateau Physiographic Region. Many of these endemic species are rare and some are endangered and include both woody and herbaceous species (Correll and Johnston 1970; Poole et al 2007; Van Auken 2018; Carr 2019).

The species examined in the present study, *Phaseolus texensis* Delgato-Salinas and Carr (Boerne Bean, Fabaceae = Leguminosae) is a relatively newly described species endemic in central Texas (Delgato and Carr 2007). It is has been found in the southeastern part of the Edwards Plateau Physiographic Region, in juniper live oak-woodlands, (Van Auken 2018).

There have been several new herbaceous species recently described from central Texas including two legumes (Delgato-Salinas and Carr 2007; Holmes and Singhurst 2008), one carrot or parsley (Apiaceae = Umbelliferae, Keith 2012), and one mustard (Brassicaceae, Turner 2012). Although the morphological characteristics of these species including flowers, fruits, leaves, and stems have been

described along with general descriptions of where they have been found, little is written about their physiological or ecological characteristics. *Phaseolus texensis* is considered endemic in Texas, found in rocky canyons of the eastern and southern part of the Edwards Plateau physiographic region of central Texas (Delgato-Salinas and Carr 2007). Although molecular phylogenetic analyses have been completed, no physiographic studies have been identified.

A plant's photosynthetic parameters affect its inherent growth rate and thus its biomass (Givnish et al, 2004; Begon et al. 2006; Valladares and Niinemets 2008; Keddy 2017). Consequently, understanding a plant's photosynthetic characteristics can help explain how a plant is adapted to environmental stress (Crowley 1997). Nevertheless, until now, there have been no studies that we could find concerning photosynthetic rates or growth rates of *Phaseolus texensis.* Photosynthetic rates have been measured for a number of the species occurring in the central Texas Edwards Plateau Physiographic Region (Van Auken and Bush 2015). Some have high photosynthetic rates suggesting they are sun species (Boeck and Van Auken 2017) and others have low photosynthetic rates indicating they are shade adapted understory species (Wayne and Van Auken 2012). While another group has photosynthetic rates that lie in-between sun and shade plants, this group may be highly variable and comprise many edge species (Van Auken and Bush 2011) or they are potentially juveniles of overstory species starting growth in the understory with the potential to reach the overstory.

Usually, if a plant is found below another plant or is in the understory it is probably a sciophyte (shade) plant, however, many tree species start their growth in the understory and then grow into the overstory. An example would be *Celtis laevigata,* a late successional dominant species, which is found under the canopy of dominant early successional species (Bush and Van Auken 1986; Van Auken and Bush 2013). Although, sometimes plants grow in the shade not because they cannot grow in the sun, but because competition from heliophytes (sun plants) is high and the heliophytes restrict them to the understory. Similar distribution patterns have been described for other species, but the distributions have been caused by differential herbivory in high light versus low light or shady environmental conditions (Louda and Rodman 1996; Marion and Crone 2006; Leonard and Van Auken 2013). It is possible that *P. texensis* leaf pigments are photo-oxidized at high light levels or overheating of the leaves occurs (Begon et al. 2006). It seems *Phaseolus texensis* grows in the understory of various species, but the reason is unknown. It could be that it is a sciophyte and the C_4 grasses out compete it in the open for sunlight or for some other resource.

PURPOSES

The purposes of the present study were to examine the light response of leaves of *Phaseolus texensis* growing in the understory of a *Juniperus ashei/Quercus fusiformis* (Ashe juniper/live oak) canopy and to compare gas exchange rates at various light levels to determine if photosynthetic rates represent a heliophyte, sciophyte, or intermediate level species.

MATERIALS AND METHODS

STUDY AREA-The plants examined were located below a *Juniperus ashei/Quercus fusiformis* canopy or overstory, on private property, near Boerne, Texas (98.6808W-29.6977N); approximately 48 km (30 miles) north of San Antonio, Texas. The field site was near the southern edge of the Edwards Plateau just north of the Balcones Escarpment in central Texas. Soils in this area are in the Crawford Series. They are stony clay in texture, and are shallow to moderately deep over hard limestone with a zero to three percent slope (USDA NRCS Accessed 2017). Soils have a non-calcareous clay surface layer which is 20-22 cm thick and a subsurface layer which contains limestone which is approximately 66 cm thick (Mollisols over limestone bedrock, SCS 1977).

The area has a mean annual temperature of 20°C with monthly means ranging from 9.6°C in January to 29.4°C in July (NOAA 2018). Mean annual precipitation is 78.7 cm, bimodal, with peaks occurring in May and September (10.7 cm and 8.7 cm, respectively), with little summer rainfall, high evaporation and high variability.

Vegetation in the area where *P. texensis* has been found consists of *Juniperus-Quercus* savanna or woodland, representative of savanna and woodlands found throughout this region, but higher in woody plant density than woodland communities farther to the west (Van Auken et al. 1979; Van Auken et al. 1980; Smeins and Merrill 1988). *Juniperus ashei* (Ashe juniper) and *Quercus fusiformis* (plateau live oak) are dominant woody species with subdominants including *Diospyros texana* (Texas persimmon) and *Sophora secundiflora* (mountain laurel). Interspersed in the woodlands are sparsely vegetated intercanopy patches or gaps (Van Auken 2000). The major herbaceous species below the canopy is usually *Carex planostachys* (cedar sedge)(Wayne and Van Auken 2008). *Aristida longiseta* (red three-awn), *Bouteloua curtipendula* (side-oats grama), *Bothriochloa laguroides* ssp. *torreyana* (silver bluestem), *B. ischaemum* var. *songarica* (King Ranch bluestem), various other C_4 grasses, and a variety of herbaceous annuals are common in the gaps. Light levels are higher in the gaps compared to woodland (Boeck and Van Auken 2017) which is also true for soil temperature (Wayne and Van Auken 2004).

GAS EXCHANGE-A Li-Cor 6400 portable photosynthetic meter was used to measure gas exchange of individual leaflets as a function of light level or photosynthetic-flux density (PFD). Measurements were made with plants fully leafed out in March and April 2020, within ± three hours of solar noon using a gas flow rate of 400 µmol/s and a CO_2 concentration of 400 µmol/mol at PFDs of 0, 5, 10, 25, 50, 75, 100, 200, 400, 600, 800, 1000, 1200, 1600, 1800 and 2000 µmol/m^2/s. Each leaflet used covered the entire chamber.

Leaflets from six plants growing below a *Juniperus ashei/Quercus fusiformis* canopy were selected and measured separately. Leaves were fully expanded on the growing vine (stem) and not newly expanded leaves. One undamaged leaflet was selected on each tri-foliate leaf for measurements. Plants in the shaded canopy understory were at light levels of 169 µmol/m^2/s with a range from < 5 to 431 µmol/m^2/s.

Maximum photosynthesis, A_{max} (µmol CO_2/m^2/s) was calculated. Photosynthetic-flux density PFD at A_{max} (µmol/m^2/s), transpiration at A_{max} (µmol H_2O/m^2/s), conductance at A_{max} (mmol H_2O/m^2/s), light saturation point (µmol/m^2/s), dark respiration (µmol CO_2/m^2/s), light compensation point (µmol/m^2/s), and the quantum yield efficiency (µmol CO_2/ µmol quanta) for each replicate was determined and then means for leaves of each treatment were calculated. Data for each replication (leaflet) was fit to the model of Prioul and Chartier (Prioul and Chartier 1977) using the PC software package Photosyn Assistant (Dundee Scientific, Dundee, Scotland). A_{max} represented the highest net photosynthesis rate. Light saturating photosynthesis depicted the PFD when the slope of the initial rate line reached the A_{max}. Dark respiration was the gas exchange rate at a PFD of 0 µmol/m^2/s (y-intercept of the line for the initial rate). The light compensation point was calculated as the PFD when the photosynthetic rate was 0 µmol CO_2/m^2/s (x-intercept of the line for the initial rate). The quantum yield efficiency was calculated using the dark value and increasing PFDs until the regression coefficient of the slope decreased.

Light response curves were generated for each leaflet. Assumptions for parametric statistics were not met, therefore the Kruskal-Wallis Test (rank sum) was used to determine if differences occurred between photosynthesis, conductance, or transpiration (Hajek 1969; SAS Institute Inc. 2017). An alpha value of 0.05 was used throughout.

RESULTS

The mean photosynthetic light response curve for *Phaseolus texensis* grown in the understory of a *Juniperus ashei/Quercus fusiformis* canopy at a light level of 169 ± 28 µmol/m^2/s is presented (Figure 1A). The function is a positive polynomial function reaching a plateau or steady state as light levels increased. The mean photosynthetic rate for the leaflets of *P. texensis* was 3.29 µmol CO_2/m^2/s ranging from -0.235 to 5.475 µmol CO_2/m^2/s over the 16 light levels measured. There were significant differences in photosynthetic rates between at least two of the light levels (Kruskal-Wallis Test, $P = < 0.0001$). Conductance and transpiration as a function of light level are shown in Figure 1B and 1C. The transpiration rate for leaflets decreased from 0.54 mmol H_2O/m^2/s at the lowest light level examined to 0.31 mmol H_2O/m^2/s at a PFD of 50 µmol/m^2/s and then increased to 0.92 mmol H_2O/m^2/s at an approximate PFD of 1000 µmol/m^2/s. There were only slight additional differences as light levels were increased to the maximum level tested (Figure 1C). Mean stomatal conductance followed a very similar trend (Figure 1B). There were significant differences in conductance or transpiration rates between at least two of the light levels (Kruskal-Wallis Test, $P = 0.0006$ and $P = 0.0003$, respectively).

Mean maximum photosynthetic rate (A_{max}) for leaflets of *P. texensis* was estimated at 5.99 ± 0.17 µmol CO_2/m^2/s (Table 1). The quantum yield efficiency or initial slope (ϕ or IS) for leaflets of *P. texensis* was 0.039 ± 0.006 µmol CO_2/µmol quanta (Table 1). The light compensation point (L_{cp}) was 0.102 ± 0.011 µmol/m^2/s, the light saturation point (L_{sp}) was 155 ± 19 µmol /m^2/s and dark respiration (R_d) was 0.235 ± 0.135 µmol CO_2/m^2/s (Table 1).

DISCUSSION

Species that are present below closed forest canopies generally have photosynthetic rates (A_{max}) that are low compared to species in the open (Zangerl and Bazzaz 1983; Hättenschwiler and Körner 1996; Hirose and Bazzaz 1998; Hull 2002). When understory plants are exposed to higher light levels more distinctive of open grassland communities or disturbed areas, gas exchange rates do not generally increase. Species from the Edwards Plateau region in central Texas savannas have responses to light levels that have been difficult to predict, probably because of recent community changes such as species encroachment or habitat manipulation.

Comparison of *Phaseolus texensis* A_{max} rate with the A_{max} of C_4 grasses in open habitats confirmed that the C_4 grasses such as *Bouteloua curtipendula* had A_{max} rates 5.26 times higher than *P. texensis* (Wayne and Van Auken 2012) (see Table 1). An open habitat herbaceous species (*Heliotropium tenellium*) had A_{max} rates that were 5.83 times higher than *P. texensis* (Table 1). Other species included a leguminosae shrub, as well as four asteraceae and a malvaceae sub-shrubs; all had intermediate photosynthetic rates and were thought to be facultative species able to grow below a canopy, at the canopy edge and sometimes in the open (Furuya and Van Auken 2009; Gagliardia and Van Auken 2009; Furuya and Van Auken 2010; Van Auken and Bush 2011). Photosynthetic rates of these species were modified by the light levels they were exposed to, but they never had A_{max} values as high as the C_4 grasses found in associated open high light habitats.

Phaseolus texensis is apparently "restricted to the eastern and southern part of the Edwards Plateau of Texas at elevations from 200 to 600 m" (Delgado-Salinas and Carr 2007). They list specific locations where it has been found and more recent sites can be observed by searching Boerne Bean (Boerne Bean 2020). Small populations have been noted in various mixed central Texas woodlands usually on limestone cliffs or outcrops and in some places along intermittent creeks (Delgado-Salinas and Carr 2007). The environments where this species has been reported appear to be low light environments below the canopy of *Juniperus-Quercus* woodlands in Central Texas savannas and not in open grasslands.

Gas exchange rates for *P. texensis* below the canopy at low light levels, (just above the light saturation point, L_{sat}) were equivalent or within the range of rates measured for true understory species (Table 1). These rates should make *P. texensis* a good competitor with other understory species in this same understory environment. Interestingly, gas exchange rates for *P. texensis* at higher light levels were within the range of other typical shade or understory species (Begon et al. 2006). Other photosynthetic parameters, including light saturation, light compensation, dark respiration, conductance, and transpiration, were within the range of values for understory or shade adapted species (Table 1). These responses are consistent with findings for shade plants, but close to values reported for facultative species (Hull 2002; Larcher 2003; Givnish et al. 2004; Valladares and Niinemets 2008; Van Auken and Bush 2015). The parameters measured for shade adapted leaves of *P. texensis* at elevated light levels did not increase significantly, suggesting that *P. texensis* is a true understory species capable of growth in low to medium light environments, but not in high light environments such as open grasslands.

In general, true understory species or shade species from eastern North American deciduous forest have A_{max} values and photosynthetic rates comparable to rates reported for *P. texensis* in the current study. No *P. texensis* plants were found in full sun, consequently we do not know if they could acclimate to a variable light environment as seen for example in light gaps such as reported for other species (Hull 2002; Valladares and Niinemets 2008).

The dark respiration rate of shade leaves of *P. texensis* growing below a *Juniperus ashei/Quercus fusiformis* canopy at light levels of 169 ± 28 μmol/m^2/s was 0.235 ± 0.135 μmolCO_2/m^2/s or about 20% of values for other species growing in the same habitat type (Hirose and Bazzaz 1998; Hull 2002; Van Auken and Bush 2015). Dark respiration for shade-adapted species is typically low due to their lower metabolism (Bjorkman 1968; Bazzaz and Carlson 1982). The respiration rate of *Polygonum pensylvanicum* a wetland plant grown in low light was ~ 0.5 μmol·CO_2/m^2/s, whereas the rate for its leaves in full sun was twice as high (Bazzaz and Carlson 1982).

Other gas exchange values reported for *P. texensis* are within the range or lower than values reported for similar shade adapted plants. For example, the quantum yield efficiency reported here was 0.039 ± 0.006 μmol·CO_2/μmol quanta, for shade leaves which is in the range of values reported for other shade species (0.035 – 0.052 μmol·CO_2/μmol quanta) (Hirose et al. 1997). This may be a rapid response to light flecks below the canopy, but this is speculation at this time. Stomatal conductance and transpiration reported for *P. texensis* in the current study were similar to other studies and indicate open stomates; however, many factors affect the levels of these parameters including temperature and soil water content (Wieland and Bazzaz 1975; Zangerl and Bazzaz 1984; Yun and Taylor 1986; Munger et al. 1987a; Munger et al. 1987b; Stafford 1989).

We never found plants of this species in open grasslands. Descriptions of this species suggest it is shade adapted (Delgado-Salinas and Carr 2007; Boerne Bean 2020). In parts of the range of the genus *Phaseolus*, some species or individuals of various species may establish and grow in low density grasslands outside of or at the edge of woodland canopies. However, all of the *P. texensis* plants that we found were below the *Juniperus-Quercus* woodland canopy.

A species found in a given habitat can tolerate or requires the conditions present in that habitat where conditions may be most favorable for its growth and survival. Nonetheless, sorting out the characteristics and levels of that or those factors can be taxing (Smith and Smith 2012; Keddy 2017). We believe that while *P. texensis* is usually found growing in shade, gas exchange characteristics may not be the only factor controlling its apparent habitat preference. The drought tolerance of this species should be compared with the drought tolerant C_4 grasses growing in the open. Another environmental factor or a combination of factors may limit the growth of *P. texensis* to shaded understory habitats including possible photo-inhibition of leaf pigments or overheating of leaves (Begon et al. 2006). Similar patterns

of distribution have been reported for other species, but restrictions were caused by differential herbivory (Louda and Rodman 1996; Maron and Crone 2006; Leonard and Van Auken 2013).

Water may be a resource limiting *P. texensis* to sub canopy positions because of greater water use efficiency by more drought tolerant C_4 grasses in open areas. This factor may keep *P. texana* restricted to canopy habitats where the C_4 grasses cannot grow because of low light levels and the high light requirements of the C_4 grasses (Wayne and Van Auken 2009). Possibly, higher soil water levels below the canopy might be available to *P. texensis* rather than to other species in this understory environment. Survival of deep rooted woody species seems to be key to their survival during extreme drought conditions, with shallow rooted woody species like *Juniperus ashei* suffering higher mortality (Johnson et al. 2018). Unfortunately, root growth and depth of penetration is unknown at this time for *P. texensis.*

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Table 1. Comparison of mean ± one se for the maximum net photosynthetic rates (A_{max}), light level (PFD) at the A_{max}, and other photosynthetic parameters for *Phaseolus texensis,* an additional shade plant and two known sun plants from central Texas are presented. Leaflets growing on *P. texensis* plants found in the understory of a *Juniperus ashei/Quercus fusiformis* canopy at a light level of 169 ± 28 µmol/m^2/s are presented.

Parameter	*Phaseolus texensis**	*Carex planostachys***	*Heliotropium tenellium****	*Bouteloua curtipendula*****
Amax-max. photo. rate	5.99 ± 0.17	4.9 ± 0.3	34.96 ± 4.43	31.6 ± 0.5
Light Level at Amax	1000	500	2000	1633
L*sat* - Light saturation	155 ± 19	151±16	591 ± 122	630 ± 78
L*cp* - Light comp. point	0.102 ± 0.011	4 ± 2	38 ± 3	58 ± 10
R*d* - Dark respiration	0.235 ± 0.135	0.4 ± 0.0	2.63 ± 0.38	3.0 ± 0.1
IS - Initial slope	0.039 ± 0.006		0.07 ± 0.01	
gs - Stomatal cond.	0.06818	0.07 ± 0.01	0.44 ± 0.08	0.25 ± 0.01

*This study
**Wayne and Van Auken (2012)
***Boeck and Van Auken (2017)
****Wayne and Van Auken (2012)

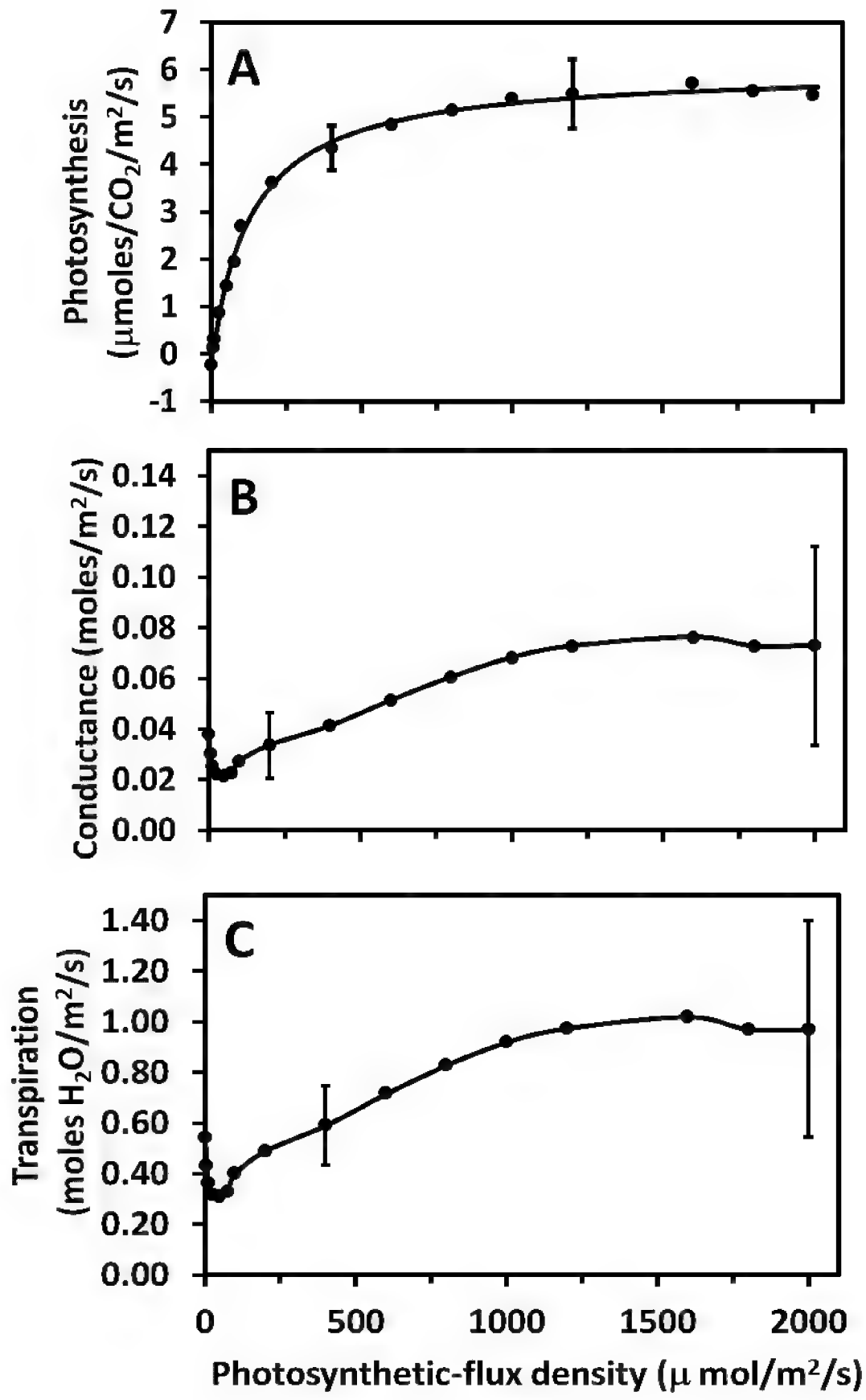

Figure 1. A) Mean photosynthetic light response curve, B) conductance, and C) transpiration for *Phaseolus texensis* measured from 0.0 µmol/m^2/sec to 2000 µmol/m^2/sec. Standard error bars are examples. Plants were in the understory of a *Juniperus ashei/Quercus fusiformis* canopy at a light level of 169 ± 28 µmol/m^2/s (mean ± se). Measurements were made on April 13 and April 14, 2020.

New combinations in *Dichanthelium* (Poaceae)

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ABSTRACT

The following new combinations are provided in *Dichanthelium* for the United States: **Dichanthelium albomarginatum** (Nash) Wipff, **Dichanthelium chrysopsidifolium** (Nash) J.R. Thomas & Wipff, **Dichanthelium concinnius** (Hitchcock & Chase) Wipff, **Dichanthelium equilaterale** (Scribner) Wipff, **Dichanthelium flavovirens** (Nash) Wipff, **Dichanthelium languidum** (Hitchcock & Chase) Wipff, **Dichanthelium mutabile** (Scribner & J.G. Smith *ex* Nash) Wipff, **Dichanthelium patentifolium** (Nash) Wipff, **Dichanthelium patulum** (Scribner & Merrill) Wipff, **Dichanthelium polycaulon** (Nash) Wipff, **Dichanthelium trifolium** (Nash) Wipff, **Dichanthelium vernale** (Hitchcock & Chase) Wipff, **Dichanthelium wilmingtonense** (Ashe) Wipff**, Dichanthelium xalapense** (Kunth) Wipff and **Dichanthelium xalapense** (Kunth) Wipff var. **strictirameum** (Hitchcock & Chase) Wipff.
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KEY WORDS: *Dichanthelium*, *Panicum*, USA.

Hitchcock and Chase (1910) established *Panicum* subgenus *Dichanthelium* and continued to recognize it as a subgenus in their later treatments of *Panicum* L. (Hitchcock 1913, 1920, 1935, 1936, 1951; Hitchcock & Chase 1915), as did Silveus (1933, 1942) and other authors. Gould (1974) elevated subg. *Dichanthelium* to the rank of genus with the support of this elevation given by Gould and Clark (1978). More recent evidence for the recognition of *Dichanthelium* has come from research involving the molecular phylogeny of Panicoideae (Giussani et al. 2001; Aliscioni et al. 2003; Morrone et al. 2008). These studies demonstrate that *Panicum* s.l. is polyphyletic unless *Dichanthelium*, among others, is treated as a separate genus. Additionally, many of the presumed intermediate taxa (*Panicum* sect. *Cordovensia* Parodi and the still unresolved *P. longipedicellatum* Swallen) that were used to maintain *Dichanthelium* as a subgenus of *Panicum* have been shown to represent distinct lineages (Aliscioni et al. 2003; Morrone et al. 2008). *Panicum* sect. *Cordovensia* has already been transferred into a separate genus (Morrone et al. 2008).

Hitchcock & Chase (1910) recognized 17 informal groups in subg. *Dichanthelium*, 109 species and 5 varieties. Radford et al. (1968) reduced the number of recognized taxa in *Panicum* subg. *Dichanthelium*. Gould and Clark (1978) made many more reductions in the number of recognized taxa, which they even admitted was "somewhat arbitrary and certainly not entirely satisfactory." Subsequent work has determined that Radford et al. (1968) and Gould & Clark (1978) had been overzealous reductions. Ladd & Thomas (2015) stated that even the treatment of "...Freckmann and Lelong (2003), does not accurately reflect the morphological entities, ecological performance, and taxonomic relationships of the group. Time and again, morphologically and ecologically distinct entities are subsumed into broad "species," in the process disenfranchising then meaningful ecological information about habitat affinities and distinctness that are of value in assessing vegetation and habitat conditions. Recent studies (e.g. Thomas 2008[, 2015]) and detailed field assessments support maintaining some narrower species concepts within the group." Since 1978, a number of the old *Panicum* subg. *Dichanthelium* names treated in synonymy by Radford et al. (1968) and Gould & Clark (1978) are now recognized as valid species, subspecies, or varieties in *Dichanthelium* (e.g. Mohlenbrock 1985, 2001, 2015; Freckmann & Lelong 2002, 2003; LeBlond 2001, 2016, 2017, 2018; Ladd & Thomas 2015; Thomas 2015, 2017, 2018; Greuter & Rankin Rodríguez 2016; Wilhelm & Rericha 2016; Wipff & Shaw

2018). More of the old *Panicum* subg. *Dichanthelium* taxa will be transferred to *Dichanthelium* as this very complex group is continually better understood.

During the preparation of the second edition of the *Guide to Texas Grasses*, Texas A&M University Press, it is necessary to make nomenclatural changes to coincide with the author's concept of the taxa involved. The *Dichanthelium* treatment for Texas will more reflect the circumscription of species as treated by Hitchcock (1951). So, distinctive taxa, long recognized at species or variety rank in *Panicum* subg. *Dichanthelium* in Hitchcock & Chase (1910) and Hitchcock (1951), are here transferred to D*ichanthelium*.

Dichanthelium albomarginatum (Nash) Wipff, **comb. nov.** BASIONYM: *Panicum albomarginatum* Nash, Bull. Torrey Bot. Club 24(1): 40-41 (1897). **TYPE: FLORIDA: Lake Co.** In vicinity of Eustis, low pine land, 1-15 June 1894, *Nash 925* (HOLOTYPE: NY-00381577, image!; ISOTYPES: NY-00381576, image!, F-0046859F, image!, K-000674410, image!, MO-2151649, image!, NDG-12260, image!, P-00740930 image!, PH-00018616 image!, US-00133064, image!, US-01117846, image!).

Dichanthelium chrysopsidifolium (Nash) J.R. Thomas & Wipff, **comb. nov.** BASIONYM: *Panicum chrysopsidifolium* Nash in J.K. Small, Fl. S.E. U.S.: 100, 1327 (1903). **TYPE: FLORIDA: Leon Co.:** Lake Jackson, 12 May 1886, *A.H. Curtiss s.n.* (HOLOTYPE: NY-00413960, image!; ISOTYPE: US-00133142, image!).

Dichanthelium concinnius (Hitchcock & Chase) Wipff, **comb. nov.** BASIONYM: *Panicum concinnius* Hitchcock & Chase, Contr. U.S. Natl. Herb. 15: 263 (1910). *Panicum gracilicaule* Nash, in J.K. Small, Fl. S.E. U.S.: 98, 1327 (1903), nom. illeg. hom., non *Panicum gracilicaule* Rendle (1899). **TYPE: Alabama: Jackson Co.:** sandy soil along a creek, Sand Mtn., 5 Jun 1900, *T.G. Harbison 2415* (HOLOTYPE: NY-00381613, image!; ISOTYPE: US-00148543, image!).

Dichanthelium equilaterale (Scribner) Wipff, **comb. nov.** BASIONYM: *Panicum equilaterale* Scribner, U.S.D.A., Bull. Div. Agrostol. 11: 42, t.2. 1898 (July). *Dichanthelium commutatum* (Schultes) Gould subsp. *equilaterale* (Scribner) Freckmann & Lelong, Sida 20(1): 169. 2002. **TYPE: FLORIDA: Lake Co.**: vicinity of Eustis, scrub hummock, 16-25 August 1894, *G.V. Nash 1674* (LECTOTYPE: US-00147824 (743929), image!; ISOLECTOTYPES: US-01117756 (208318), image!; US-01117758 (480517), image!; US-01117757 (823254), image!; GH-00024100, image!; MICH-1108734, image!; NY-00413980, image! - designated by Hitchcock & Chase, Contr. U.S. Natl. Herb. 15: 310 (1910). SYNTYPE: FLORIDA: Lake Co.: vicinity of Eustis, high pine land, 16-30 June 1894, *G.V. Nash 1120* (K-000674484, image!; US).

Dichanthelium flavovirens (Nash) Wipff, **comb. nov.** BASIONYM: *Panicum flavovirens* Nash, Bull. Torrey Bot. Club 26: 572 (1899). **TYPE: FLORIDA: Lake Co.**: Eustis Lake, 16-30 Jun 1895, *G.V. Nash 2061* (HOLOTYPE: NY-00381599, image!; ISOTYPES: NY-00381600, image!, US-00148543, image!, GH-00024103, image!, NDG-06846, image!, US- 00147840, image!).

Dichanthelium languidum (Hitchcock & Chase) Wipff, **comb. nov.** BASIONYM: *Panicum languidum* Hitchcock & Chase, Contr. U.S. Natl. Herb. 15: 232, f. 245 (1910). *Panicum unciphyllum* forma *prostratum* Scribner & Merrill, Rhodora 3: 124. 1901, non *Panicum prostratum* Lamarck (1791). **TYPE: MAINE: YORK CO.,** South Berwick, dry open woods, 26 September 1897, *M.L. Fernald s.n.* (HOLOTYPE: US-00148071, image!; ISOTYPES: NEBC-00028648, image!, NEBC-00028647, image!).

Dichanthelium mutabile (Scribner & J.G. Smith *ex* Nash) Wipff, **comb. nov.** BASIONYM: *Panicum mutabile* Scribner & J.G. Smith *ex* Nash, in J.K. Small Fl. Southeast. U.S. 103, 1327. 1903 (22 July). **TYPE: MISSISSIPPI:** Biloxi, 15 September 1896, *S.M. Tracy 3074* (HOLOTYPE: NY-00381646, image!; ISOTYPE: US-00132994, image!).

Dichanthelium patentifolium (Nash) Wipff, **comb. nov.** BASIONYM: *Panicum patentifolium* Nash, Bull. Torrey Bot. Club 26(11): 574-575 (1899). **TYPE: FLORIDA: Lake Co.**: vicinity of Eustis, scrub hummock, 12-31 March 1894, *Nash 72* (HOLOTYPE: NY-00381658, image!; ISOTYPES: NY-00381659, image!, G-00176626, image!, GH-00024111, image!, MO-440032, image!, NDG-06613, image!, US-00147942, image!).

Dichanthelium patulum (Scribner & Merrill) Wipff, **comb. nov.** BASIONYM: *Panicum nashianum* var. *patulum* Scribner & Merrill, U.S.D.A. Circ. Div. Agrostol. 27: 9 (1900). *Panicum patulum* (Scribner & Merrill) Hitchcock, Rhodora 8(95): 209 (1906). *Panicum lancearium* Trinius var. *patulum* (Scribner & Merrill) Fernald, Rhodora 36: 80 (1934). *Dichanthelium sabulorum* (Lamarck) Gould & C.A. Clark var. *patulum* (Scribner & Merrill) Gould & C.A. Clark, Ann. Missouri Bot. Gard. 65: 1113 (1979). *Panicum sabulorum* Lamarck var. *patulum* (Scribner & Merrill) C.F. Reed, Phytologia 67: 452 (1989). *Dichanthelium portoricense* (Desvaux *ex* Hamilton) B.F. Hansen & Wunderlin subsp. *patulum* (Scribner & Merrill) Freckmann & Lelong, Sida 20: 170 (2002). **TYPE: FLORIDA: Manatee Co.**: Braidentown [Bradenton], in fertile woods along the Manatee, not frequent, 3 September [October] 1898, *R. Combs 1296* (HOLOTYPE: US-00132997, image!).

Dichanthelium polycaulon (Nash) Wipff, **comb. nov.** BASIONYM: *Panicum polycaulon* Nash, Bull. Torrey Bot. Club 24(4): 200-201 (1897). **TYPE: FLORIDA: Hillsborough Co.**: Tampa, flatwoods, 20 Aug 1895, *G.V. Nash 2420a* (HOLOTYPE: NY-00381668, image!; ISOTYPE: US-00139880, image! (photo of holotype and spikelets in a fragment packet).

Dichanthelium trifolium (Nash) Wipff, **comb. nov.** BASIONYM: *Panicum trifolium* Nash, Bull. Torrey Bot. Club 26(11): 580 (1899). **TYPE: GEORGIA: Bibb Co.**: in the Ocmulgee River Swamp, below Macon, 18-24 May 1895, *J.K. Small s.n.* (HOLOTYPE: NY-00381691, image!; ISOTYPE: US-00140076, image!).

Dichanthelium vernale (Hitchcock & Chase) Wipff, **comb. nov.** BASIONYM: *Panicum vernale* Hitchcock & Chase, Contr. U.S. Natl. Herb. 15: 266, f. 293 (1910). **TYPE: FLORIDA: Columbia Co.:** Lake City**,** sphagnum bog, 16 April 1906, *A.S. Hitchcock 1020* (HOLOTYPE: US-00148085, image!; ISOTYPES: GH-00024115, image!, ISC-v-0000582, image!).

Dichanthelium wilmingtonense (Ashe) Wipff, **comb. nov.** BASIONYM: *Panicum wilmingtonense* Ashe, J. Elisha Mitchell Sci. Soc. 16(2): 86 (1899 publ. 1900). **TYPE: NORTH CAROLINA: New Hanover Co.:** Shady slopes on the sand hills one mile to north of Wilmington, 17 May 1899, *W.W. Ashe s.n.* (HOLOTYPE: NCU-00000396, image!, ISOTYPE: US-00148099, image!)

Dichanthelium xalapense (Kunth) Wipff, **comb. nov.** BASIONYM: *Panicum xalapense* Kunth in F.W.H. von Humboldt, A.J.A. Bonpland & C.S. Kunth, Nov. Gen. Sp. (quarto ed.) 1: 103 (1815[1816]). **TYPE: MEXICO: Veracruz**: near Jalapa, "in regno Mexicano prope xalapa, regione temperata." February, *F.W.H.A. von Humboldt, & A.J.A. Bonpland s.n.* (HOLOTYPE: P-00128859, image!; ISOTYPE: P-0012886, image!, US-00140093, image! (2 spikelets)).

Dichanthelium xalapense (Kunth) Wipff var. **strictirameum** (Hitchcock & Chase) Wipff, **comb. nov.** BASIONYM: *Panicum xalapense* Kunth var. *strictirameum* Hitchcock & Chase, Contr. U.S.

Natl. Herb. 15: 161 (1910). **TYPE: MISSISSIPPI:** Jackson, 28 April 1906, *A.S. Hitchcock 1311* (HOLOTYPE: US-00148102, image!; ISOTYPE: GH-00023524, image!).

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Infrageneric nomenclature adjustments in *Crataegus* L. (Maleae, Rosaceae)

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ABSTRACT

Until recently, classification of *Crataegus* (Maleae, Rosaceae) has been mostly based on morphological data. Phenetic and cladistic approaches allowed taxonomists to establish classifications of the genus at the levels of sections and series, but without revealing clear phylogenetic relationships between these infrageneric groups. Molecular studies suggest the existence of major evolutionary lineages, some of which correspond to previously published subgenera (*C.* subg. *Americanae* and subg. *Sanguineae*). The present paper aims to complete the subgeneric classification of *Crataegus* by raising *C.* sect. *Mespilus* and sect. *Brevispinae* to subgenera. Also, in order to depict current knowledge of the phylogenetic relationships within *C.* subg. *Sanguineae*, a new *C.* sect. *Salignae* is described. In addition, we provide a new description of *Crataegus* and keys to distinguish it from other related *Maleae* genera, to determine the subgenera and, within *C.* subg. *Sanguineae*, to determine the sections. In conclusion, we summarize the current classification of *Crataegus*, excluding nothosubgenera and nothosections, in relation to the phylogeography and leaf venation patterns of the genus. Published on-line www.phytologia.org *Published on-line www.phytologia.org Phytologia 102(3): 177-199. (Sept 21, 2020). ISSN 030319430.*

KEY WORDS: *Crataegus Salignae* sect. nov., *Crataegus Brevispinae* stat. nov., *Crataegus Mespilus* stat. nov., identification, phylogeography

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Crataegus L. (Rosaceae Juss., subfam. Amygdaloideae Arn., tribe Maleae Small) is a well-defined genus including over 200 species (Phipps, 2015) that mainly occur throughout the temperate zone of the Northern Hemisphere in high light intensity habitats with hydrological regimes permitting the growth of woody trees. Some species are cultivated as ornamentals, or for their fruit. The flowers, fruit, and foliage are also the sources of natural health products (Edwards et al., 2012). *Crataegus* taxonomy is considered complicated and has attracted the attention of researchers seeking to provide a solid basis for its classification. J. C. Loudon (1838) proposed the first infrageneric divisions for the genus. He noted how the number of "sorts" of hawthorns had more than doubled since the turn of the century and explicitly chose to throw them "…into natural groups, according to the majority of their points of resemblance…," rather than preparing a technical key to sections; this was supplied instead as an appendix by the horticulturalist George Gordon (Loudon, 1838). Loudon's natural classification of *Crataegus* into 14 sections (as now understood) provided the basis for subsequent workers to deal with the rapid increase in the number of hawthorn species described through the first half of the twentieth century.

By the end of 1980s, the number of groups/sections/series had been nearly doubled. Most of the treatments employed a hierarchy with just a single level between genus and species, either sections (Schneider, 1906; Palmer, 1925; Cinovskis, 1971) or series (Rehder, 1940; Palmer, 1952; Kruschke, 1965; Rusanov, 1965). The first multilevel infrageneric classification of the whole genus was published by J. B. Phipps (1983), who grouped series into sections. At that time the division of the genus into two subgenera (*C.* subg. *Crataegus* and *Americanae* El Gazzar) by El-Gazzar (1980) was, besides being recognized as being based in part on faulty data, a nomenclatural act of little immediate significance. Rather, classification of *Crataegus* at the level of sections and series was well established as a means of organizing the morphological diversity seen within the genus (Christensen, 1992; Lance, 2014; Phipps, 2015). However, while phenetic and cladistic analyses of *Crataegus* morphological data corroborated the existence of groups, the latter failed to demonstrate definitive phylogenetic relationships (Phipps, 1983; Christensen, 1992; Dickinson and Love, 1997; Phipps, 1999).

Since then, molecular phylogenies have demonstrated greater success in elucidating cladistic relationships between sections. Molecular studies (Lo et al., 2007; Lo et al., 2009a; Lo et al., 2009b; Zarrei et al., 2014; Zarrei et al., 2015) revealed the main lines of evolution in the genus, which, it turned out, corresponded partially to the distinctions recognized by El-Gazzar (1980). Lo et al. (2009a) analyzed a sufficiently wide sample of species to be able to delineate clades corresponding not only to El-Gazzar's subgenera but also to the one subsequently described as *C.* subg. *Sanguineae* Ufimov (Ufimov, 2013). We have completed the subgeneric classification of *Crataegus* by raising two further sections to subgenera. In addition, in recognition of the cladistic relationships within *C.* subg. *Sanguineae* (Zarrei et al., 2015) we describe one further section of the genus.

OBJECTIVES

We provide a comprehensive subgeneric classification for the genus *Crataegus* in order to facilitate communication and help focus research attention on the most challenging taxonomic problems, such as relationships within *C.* subg. *Americanae* and *Crataegus*. In addition, we also describe *C.* sect. *Salignae* T.A.Dickinson & Ufimov sect. nov. in order to accommodate *C.* ser. *Cerrones* J.B.Phipps in a way that reflects its position within *C.* subg. *Sanguineae*, namely as sister group to *C.* sect. *Douglasianae* Rehder ex C.K. Schneid.[1] and sect. *Sanguineae* Zabel ex C.K.Schneid. Finally, we interpret this classification in light of the phylogeny on which it is based, using data from leaf venation that may be relevant to the future interpretation of fossils, and the (limited) fossil data that are currently available.

MATERIALS AND METHODS

We illustrate the phylogenetic relationships between the infrageneric groups that we discuss using a result from an earlier work (Fig. 1; Lo and Donoghue, 2012) and data from a recent study (Fig. 2; Liston et al. in prep.; used with permission) in which whole plastome DNA alignments were obtained from 14 diploid *Crataegus* accessions and aligned to the *Malus* ×*domestica* 'Golden Delicious' plastome sequence (Velasco et al., 2010). Relationships between these accessions were summarized as a maximum likelihood tree (RAxML; Stamatakis, 2014), rooted using the apple reference plastome. This tree was then collapsed to show just the relationships between five subgenera (and the three sections in *C.* subg. *Sanguineae*; Table 1) that are of interest here, using the function **makeCollapsedTree** in the R package TREESPACE (Jombart et al., 2017). We project this tree onto a north polar projection of a

[1] According to Art. 21.2 and Art. 32.1 of the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018) the sectional name *Douglasii* was not validly published by Loudon (1838: 823) as it is a noun in the genitive singular. The articles mentioned do not allow simple correction, so the earliest valid publication of a section containing *C. douglasii* is that of C. K. Schneider (1906: 775), and his name is the correct one that we use here.

tectonic plate reconstruction for 37 Ma produced using the ODSN Plate Tectonic Reconstruction Service (Hay et al., 1999; https://www.odsn.de/odsn/services/paleomap/paleomap.html) in order to show informally the present-day biogeographic relationships between the terminals. 37 Ma was chosen as the approximate time of diversification of the 'Crataegus' clade at Eocene-Oligocene boundary (Lo and Donoghue, 2012).

Although the main purpose of this paper is to publish new names needed to complete the infrageneric classification of *Crataegus*, we also wish to document leaf venation, a little-studied aspect of morphological variation across the subgenera, and one that is critical for identification of fossil leaf material. Leaves from specimens in the Green Plant Herbarium of the Royal Ontario Museum (TRT; Table 1) were imaged with x-rays on Kodak Industrex M100 x-ray film using a Hewlett Packard Faxitron Model K43805 (Ross, 2008) and digitized from the x-ray negative using a Hasselblad H5D-200c MS or similar camera. The original x-ray film images are negatives, with veins in white against a bluish background. "Positive" images (venation dark, against a light background) were produced using the "negative" functions of image processing software for the Macintosh™ computer (ToyViewer v5.5, Ogihara, 2014; all other images reproduced here were produced using Adobe PhotoShop™ and Pixelmator Pro™). Access to an online taxonomic database of these and additional downloadable *Crataegus* leaf venation images (Dickinson et al., 2020) is made possible by MorphoBank (O'Leary and Kaufman, 2011, 2012).

We also refer to our own field observations and the field photographs of others, as well as to published illustrations, in order to incorporate additional morphological data, notably concerning the proleptic or sylleptic growth of lateral short shoots in the '*Amelanchier*+*Crataegus*' clade (clade A in Fig. 1). Sylleptic and proleptic growth are understood as they are described by Hallé et al. (1978, p. 42 ff.).

RESULTS

The genera of the Maleae together with the genus *Gillenia* Moench form a clade (Fig. 1; Potter et al., 2007; Lo and Donoghue, 2012) that can be referred to as supertribe Pyrodae C.S.Campb., R.C.Evans, D.R.Morgan & T.A.Dickinson. Within the Pyrodae fruit type is heterogeneous, the four basal genera having dry dehiscent fruits, while the remainder of this clade (subtribe Malinae Reveal) has fleshy fruits developing from flowers that are epigynous (perigynous in *Dichotomanthes* Kurz; Rohrer et al., 1994). Within the Malinae, composite fruit walls (lignified endocarp, fleshy epicarp, as in *Prunus* L.) occur repeatedly, so as to make up tribe Crataegeae Koehne (Kalkman, 2004; Kalkman excludes *Dichotomanthes* from the Crataegeae on the grounds that its fruit is an achene partially enclosed by an accrescent hypanthium). However, the Crataegeae (named genera with black dots, Fig. 1) is clearly not monophyletic as the component genera are distributed in each of Malinae clades A, B, and C (Fig. 1) as well as in the two genera found in trichotomies (*Pyracantha* M.Roem., *Osteomeles* Lindl.; Fig. 1). The remaining genera (not listed in Fig. 1) in clades A[2], B[3], and C[4] have berry-like fruits (the *Cydonia* group and tribe Maleae in Kalkman, 2004).

All the subgenera of *Crataegus* are monophyletic (Fig. 2; Liston et al., in prep.). *Crataegus* subg. *Brevispinae* (Beadle) Ufimov & T.A.Dickinson and *Mespilus* (L.) Ufimov & T.A.Dickinson are monotypic; *C.* subg. *Americanae* and *Sanguineae* were each represented by multiple accessions in the original analysis by Liston et al. (in prep.). *Crataegus* subg. *Crataegus* has been shown to be

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[2] *Amelanchier* Medik., *Malacomeles* (Decne.) Decne., *Peraphyllum* Nutt.
[3] *Aria* (Pers.) Host, *Aronia* Medik., *Chaenomeles* Lindl., *Cydonia* Mill., *Docynia* Decne., *Docyniopsis* Koidz., *Eriolobus* (DC.) M.Roem., *Malus* Mill., *Pourthiaea* Decne., *Pseudocydonia* (C.K.Schneid.) C.K.Schneid.
[4] *Cormus* Spach, *Eriobotrya* Lindl., *Heteromeles* M.Roem., *Micromeles* Decne., *Photinia* Lindl., *Pyrus* L., *Rhaphiolepis* Lindl., *Sorbus* L. s. str.

monophyletic elsewhere (Lo et al., 2010; Lo and Donoghue, 2012). *Crataegus* ser. *Cerrones* has been shown to be monophyletic and sister to one or both of *C.* sect. *Douglasianae* and *Sanguineae* (Lo et al., 2010; Zarrei et al., 2014), and so warrants placement in *C.* subg. *Sanguineae* in its own section, *C.* sect. *Salignae*. The subgenera we recognize can also be seen to differ to some extent in their patterns of secondary venation (Fig. 3). The festooned semicraspedodromous secondary venation of *C.* subg. *Brevispinae* (*C. brachyacantha*; Fig. 3a) is also seen in *Hesperomeles* Lindl. (online image, Kelly, 2008), the sister genus of *Crataegus* (Li et al., 2012; Liu et al., 2020). *Crataegus* subg. *Mespilus* appears to be unique in its reticulodromous secondary venation (*C. germanica*; Fig. 3b). The remainder of the genus exhibits mostly craspedodromous or semicraspedodromous secondary venation (Fig. 3c–j; Dickinson et al., 2020). Sylleptic development of short shoot vegetative increments occurs not only in *Amelanchier* (Fig. 8a, Phipps 2016a) but apparently also in *Malacomeles* (Velazco-Macias, 2014) and *Peraphyllum* (Boone, 2002–onwards; Campbell, 2015), in that these latter images appear to show two coeval shoots developing, one reproductive and more advanced, and the other vegetative.

TAXONOMY

We provide a new description for *Crataegus* in the currently accepted circumscription as well as descriptions of the new subgenera and section. We also provide keys to distinguish *Crataegus* from some other genera in Maleae, a key to determine subgenera, and a key to determine sections in *C.* subg. *Sanguineae*.

***Crataegus* L., Sp. Pl., 1: 475. 1753, nom. cons.** (Talent et al., 2008; Brummit, 2011; Barrie, 2011).

= *Mespilus* L., Sp. pl., 1: 478. 1753.

= *Oxyacantha* Medik., Phil. Bot., 1: 150. 1789.

= *Azarolus* sensu M.Roem., Fam. nat. syn. monogr. 3: 132. 1847, non *Lazarolus* Medik., Phil. Bot., 1: 134. 1789.

= *Halmia* Medik. ex M.Roem., Fam. nat. syn. monogr. 3: 134. 1847.

= *Anthomeles* M.Roem., Fam. nat. syn. monogr. 3: 140. 1847.

= *Phaenopyrum* M.Roem., Fam. nat. syn. monogr. 3: 152. 1847. ≡ *Gymnomeles* Oerst., Vidensk. Meddel. Dansk Naturhist. Foren. Kjöbenhavn, 1859: 111. 1860, nom illeg. ≡ *Phalacros* Wenz., Linnaea, 38, 1: 164. 1874, nom. illeg.

= *Polyomeles* Oerst., Vidensk. Meddel. Dansk Naturhist. Foren. Kjöbenhavn, 1859: 111. 1860.

= *Symphyomeles* Oerst., Vidensk. Meddel. Dansk Naturhist. Foren. Kjöbenhavn, 1859: 111. 1860.

Type (lectotype, designated by W. W. Eggleston in Britton and Brown, 1913: 294): *C. oxyacantha* L. nom. utique rej. (Lambinon, 1981; Brummitt, 1986; Voss, 1987) (= *C.rhipidophylla* Gand.).

Shrubs and polycormic or monocormic trees up to 10–15 m tall. Resting buds subglobose or ovoid, sometimes subconical, rarely conical, indumentum more or less the same as on the twigs. Twigs of the current year epruinose, rarely pruinose, glabrous or more or less pubescent to densely tomentose, lanate or villous. Young twigs (of the previous years) variable in color from grey, brown and reddish to yellow and orange. Mature bark greyish or brownish, sometimes more or less orange, platelike, exfoliating in small, angular scales. Aphyllous thorns present at least on some shoots, variable in length (1–10 cm), curvature, stoutness and color. Spine-tipped, leafy short shoots (leafy thorns, as in *Pyracantha*, Fig. 26 in Phipps, 1983) present or absent. Branched thorns may be present on mature trunks. Long (extension) shoots present, sterile short shoots present or not. Leaves deciduous, sometimes winter-persistent, alternate, in spiral phyllotaxy, simple, separated by internodes 2—3 cm long on long shoots, more or less crowded on short shoots (internodes often < 0.5 cm), glabrous or pubescent, microphylls, notophylls, or mesophylls (for the explanation of terms see Ellis et al., 2009); stipules caducous or persistent, free, falcate, margins entire to finely serrate, glandular or eglandular; petioles present, sometimes glandular; leaf blades often more variable in shape on long shoots than on short shoots, unlobed or more or less lobed to deeply incised, more or less narrowly to broadly ovate, elliptic, or obovate, margins entire, serrate, crenate, or dentate, teeth sometimes gland-tipped; secondary venation reticulodromous or weakly brochidodromous,

craspedodromous or semi-craspedodromous, in some cases approaching camptodromous. Inflorescence terminal (on few-leaved short flowering shoots, which arise from the resting buds on short, long, or flowering shoots of previous year), 1–50-flowered, sympodial, corymbose, umbellate, or flowers solitary; bracts sometimes present, leafy; bracteoles caducous or persistent, symmetric or falcate/stipuliform, with entire or glandular-serrate/dentate margin; pedicels present, pedicels and peduncles glabrous or pubescent, their indumentum similar to that of the twigs. Hypanthium more or less obconic, constricted apically, glabrous or pubescent, its indumentum usually similar to that of the inflorescence, but can be quite different. Indumentum of young twigs, leaves, inflorescence, and hypanthium tends to change over time and can disappear when fruits are mature. Inner surface of the free portion of the hypanthium nectar-secreting. Perianth and androecium epigynous, inserted on the rim and inner portion of the free portion of the hypanthium; ovary inferior; sepals 5, triangular, entire or more or less glandular-serrate/dentate, usually persistent, rarely caducous (e.g. *C. phaenopyrum* (L.f.) Medik.), usually shorter than the petals; petals 5, white, rarely pale cream or pinkish, more or less orbicular or elliptic, base barely clawed, apex rounded or notched; stamens 5–45, usually shorter than petals, anthers variable in color from white, cream, and pink to reddish, or purple; carpels 1–5 (–6), adnate to hypanthium and more or less fully connate; styles 1–5 (–6), free or more or less connate/touching, usually persistent, attached to pyrenes apically or more or less laterally, exserted or emerging through hypanthial disc; ovules 2 per locule, superposed, with an obturator at the bases of the two funiculi. Both ovules are fertile, but only the micropyle of the lower one is adjacent the obturator, so that only very exceptionally (<< 0.1%) is the upper ovule fertilized and also develops into a seed. Mature fruits ellipsoidal, orbicular, or pyriform polypyrenous drupes, up to 4 cm in diameter, varying in color from brown, greenish, yellow and orange, to red, bluish/purplish, and black, glabrous or pubescent; grit cells absent to abundant; hypanthial opening narrow to broad, mature hypanthial disc well developed, undulating and firm or reduced to a remnant disc, pyrene apices covered by its tissue or not so, and exposed; carpels woody; pyrenes 1–5 (–6), with one seed each, dorsally grooved, plane or more or less pitted/eroded/excavated/sulcate on ventral/radial surfaces, hypostyle glabrous or pubescent.

Key to genera in Rosaceae tribe Maleae with fruits drupaceous or drupe-like (*Crataegus*, *Chamaemeles*, *Dichotomanthes*, *Hesperomeles*, *Osteomeles*, *Cotoneaster*, *Pyracantha*) **and, within the '*Amelanchier+Crataegus*' clade (Fig. 1, clade A), the other genera lacking such fruits** (*Amelanchier*, *Peraphyllum*, *Malacomeles*). Clade attributions (A–C) refer to the phylogeny based on plastid loci (Fig. 1 here; left side of Fig. 1 in Lo and Donoghue, 2012).

1. Flowers perigynous. Ovary superior, unicarpellate, free from hypanthium, but hypanthium persistent and fleshy at maturity. Fruit an achene but appearing functionally drupaceous because of the accrescent hypanthium. 2 collateral ovules per locule, 1 seed per achene. Thorns absent. China. ..... ***Dichotomanthes* (clade B)**

— Flowers epigynous. Ovary inferior (hypanthial), 1–5 (–6)-carpellate. Fruit fleshy. Thorns present on at least some shoots or absent. 2

2. Leaves compound, pinnate, leaflets entire. Ovary inferior, 1–5-carpellate, 1 ovule per locule. Fruit drupaceous. China and Pacific islands. ..... ***Osteomeles* (in a trichotomy with clades B and C)**

— Leaves simple crenate, serrate, dentate, or entire, lobed or unlobed. 3

3. Lateral inflorescence-bearing short shoots develop syllepticallly. Fruits baccate, endocarp not lignified. Ovary inferior or semi-inferior, carpels 1–5 with additional false partitions, thus fruit 4–10-loculed. 4

— Lateral inflorescence-bearing short shoots may develop proleptically. Fruits drupaceous, seeds contained within thick-walled, lignified endocarps (pyrenes) that are themselves enclosed in a more or less fleshy pericarp. 6

4. Leaves drought-deciduous or persistent. Thorns absent. Texas, Mexico, Central America. .....***Malacomeles* (clade A)**

— Leaves winter-deciduous. 5

5. Leaves faintly and scarcely serrate, subentire or entire. Leaf blades more or less narrowly elliptic to oblanceolate or linear. Inflorescence reduced, few-flowered. Carpels 2–3. Mature fruits yellow-orange. Western USA. .....***Peraphyllum* (clade A)**
— Leaves serrate or dentate, sometimes doubly, often only in the distal 1/2 or 1/3, rarely almost entire. Leaf blades elliptic, oval, ovate, obovate, more or less oblong, or orbiculate. Inflorescence usually 5–15-flowered, rarely number of flowers is less than 5. Carpels 2–5. Mature fruits pinkish or brownish to bluish, purple or black. Eurasia, north Africa, North America. .....***Amelanchier* (clade A)**
6. Ovary unicarpellate, with 2 collateral ovules, 1 seed per pyrene (achene). Mature fruits white. Madeira.....***Chamaemeles* (clade B per Li et al., 2012)**
— Carpels (1–) 2–5. Mature fruits orange or red to black. 7
7. Leaves entire. Carpels not connate, basal 2/3 adnate. 2 collateral ovules per locule, 1 seed per pyrene. Thorns absent. Eurasia. .....***Cotoneaster* (clade C)**
— At least some leaves more or less crenate-dentate or serrate, rarely subentire. Thorns present. 8
8. Ovules usually 1 per locule, rarely 2, if 2 then superposed, 1 seed per pyrene. Central and South America. .....***Hesperomeles* (clade A per Li et al., 2012; Liu et al., 2020)**
— Ovules usually 2 per locule. 9
9. Leaves deciduous, sometimes winter-persistent. Carpels mostly connate and adnate. Ovules superposed, pyrenes typically single-seeded. North America, Eurasia. .....***Crataegus* (clade A)**
— Evergreen. Carpels half adnate and not connate. Ovules collateral. Eurasia. .....***Pyracantha* (unresolved; in a trichotomy with clade A and the clade comprising *Osteomeles* and clades B and C)**

***Crataegus* subg. *Mespilus* (L.) Ufimov & T.A.Dickinson, stat. nov.**

Basionym: *Mespilus* L., Sp. pl. 1: 478. 1753. ≡ *Crataegus* sect. *Mespilus* T.A.Dickinson & E.Y.Y.Lo in E.Y.Y.Lo, Stefanović et T.A.Dickinson, Syst. Bot., 32, 3: 609. 2007.

Type: *M. germanica* L. (lectotype, designated by M. L. Green in Hitchcock and Green, 1929: 158).

Single species *C. germanica*. This species appears to be sister to the rest of the genus, or to all of the genus except for *C. brachyacantha* (Lo et al., 2007), or to all of the genus except for *C.* subg. *Crataegus* (Liu et al., 2019; Liu et al., 2020; Liston et al., in prep.).

***Crataegus* subg. *Brevispinae* (Beadle) Ufimov & T.A.Dickinson, stat. nov.**

Basionym: *Crataegus* [unranked] *Brevispinae* Beadle in Small, Fl. S.E. U.S.: 532. 1903. ≡ *Crataegus* sect. *Brevispinae* (Beadle) C.K.Schneid., Ill. Handb. Laubholzk., 1: 791. 1906. ≡ *Crataegus* ser. *Brevispinae* (Beadle) Rehder, Man. Cult. Trees, ed. 2: 366. 1940.

Type: *C. brachyacantha* Sarg. & Engelm.

Single species *C. brachyacantha*. This species appears to be sister to the rest of the genus, or to all of the genus except for *C. germanica* (Lo et al., 2007), or to all of the genus except for *C.* subg. *Crataegus* (Liston et al., in prep.).

Key to subgenera in *Crataegus*

1. Leafy thorns present. Aphyllous thorns less than 15 mm long. Stipules usually persistent, rarely caducous (*C. germanica*), eglandular or inconspicuously glandular. Leaf margins serrate, crenate or entire. 2
— Leafy thorns absent. Aphyllous thorns usually more than 15 mm long, often more than 20 mm long. Rarely thorns can bear buds and reduced, caducous leaves. Stipules caducous or persistent, if persistent then conspicuosly glandular-serrate. Leaf margins serrate; secondary venation craspedodromous or semicraspedodromous; teeth with principal veins. 4
2. Leaf blades of short and flowering shoots more or less lobed, sometimes only shallowly to almost unlobed (e.g. *C. laevigata* (Poir.) DC. Fig. 3d, *C. cuneata* Siebold & Zucc.), very rarely unlobed (e.g. *C. scabrifolia* (Franch.) Rehder), margin more or less serrate and never entire; teeth usually with a principal vein (Fig. 3c, d). Leaf blades of long shoots usually more or less lobed, very rarely unlobed. Each lobe with a secondary vein conspicuously reaching its apex; other secondary veins often reach

apices of teeth, especially in the distal 1/3 of lamina; single secondary veins leading to nadirs of sinuses present (secondary venation craspedodromous or semicraspedodromous; Fig. 3c, d). Mature fruits varying in color from yellow to red, purple, and black. Pyrenes sulcate or plane on ventral/radial surfaces. ..... **subg. *Crataegus***

— Leaf blades of short and flowering shoots unlobed with finely crenate-serrate, serrate or entire margins, their secondary veins not conspicuously reaching the apices of teeth, but rather forming nodes just below the sinuses between them. Leaf blades of long shoots unlobed or more or less lobed; if lobed, secondary veins reaching the tips of lobes and teeth sometimes present, single secondary vein leading to nadirs of sinuses sometimes present. Mature fruits brown or bluish/purplish black. Pyrenes plane on ventral/radial surfaces. 3

3. Aphyllous thorns recurved. Resting buds subglobose or ovoid. Stipules more or less persistent, especially on long shoots. Leaves glossy; leaf blades of flowering and short shoots up to 3 cm long. Teeth of leaves of flowering and short sterile shoots present, lacking a principal vein (Fig. 3a); secondary venation festooned semicraspedodromous. Inflorescence multi-flowered. Sepals considerably shorter than petals. Post-mature petals more or less orange. Stamens 20. Mature fruits up to 15 mm in diameter, bluish or purplish black, hypanthial opening narrow (10–30% of width of fruit); pyrenes not covered by tissue of hypanthial disc. ..... **subg. *Brevispinae***

— Aphyllous thorns straight. Resting buds conic. Stipules caducous. Leaves not glossy, abaxially pilose; leaf blades up to 12 cm long. Teeth of leaves of flowering and short sterile shoots absent (Fig. 3b), or present with a small principal vein; secondary venation reticulodromous. Inflorescence 1–2-flowered. Sepals are equal or longer than petals. Post-mature petals pale brown. Stamens 20–40. Mature fruits up to 40 mm in diameter, brown, hypanthial opening wide (50–90% of width of fruit); pyrenes covered by tissue of hypanthial disc unless fruit cracks. ..... **subg. *Mespilus***

4. Considerable proportion of stipules persistent, especially on long shoots. Stipuliform, falcate bracteoles present. Leaves lobed to varying extents; secondary veins of leaves of flowering and short sterile shoots leading to sinus nadirs present (Fig. 3h) or not (Fig. 3e–g, i, j). Pyrenes strongly pitted on ventral/radial surfaces. ..... **subg. *Sanguineae*** (sect. *Sanguineae*)

— Stipules usually caducous, but sometimes persistent on long shoots. Stipuliform, falcate bracteoles absent. Secondary veins of leaves of flowering and short sterile shoots leading to sinus nadirs absent. Pyrenes plane, eroded or pitted on ventral/radial surfaces. 5

5. Mature fruits black, purplish black or purple. ... **subg. *Sanguineae*** (sect. *Douglasianae*, sect. *Salignae*)

— Mature fruits usually red, sometimes yellow, orange, pinkish or green. ..... **subg. *Americanae***

***Crataegus* sect. *Salignae* T.A.Dickinson & Ufimov, sect. nov.**

Type: *Crataegus saligna* Greene

Shrubs or small trees up to 5 m tall; thorns 15–30 (40) mm long, more or less straight, slender, 1.5–3.5 mm in diameter at the base; young shoots of the current year glabrous or sparsely pubescent, mature shoots of the previous year vary from reddish brown to red purple, older branches gray or copper-colored. Leaf blades of flowering and short shoots (notophylls-) microphylls, vary from lanceolate and oblanceolate to more or less elliptic or rhombic-elliptic, 20–60 mm long and (10)15–40 mm wide, glabrous at maturity, unlobed (Fig. 3j) or sparsely lobed, sinuses shallow. Inflorescence 5–10(15)-flowered. Pedicels, peduncles and hypanthia glabrous. Sepals entire, 1.0–1.5 mm long, stamens 20, anthers cream, and styles 4–5 (*C. saligna*), or sepals more or less glandular-serrate, 3.5–4.0 mm long, stamens 10, anthers pink, and styles 3–5 (*C. erythropoda* Ashe, *C. rivularis* Nutt. ex Torr. & A.Gray). Fruit purple to black (diameters of dry fruits in mm: *C. saligna*, 5–6.5; *C. rivularis*, 6.5–8.5; *C. erythropoda*, 7.5–8.5).

Crataegus sect. *Salignae* is distinguished by its fruit color from the red-, orange-, and yellow-fruited members of *C.* sect. *Sanguineae* (*C.* ser. *Sanguineae* (Zabel ex C.K.Schneid.) Rehder and ser. *Altaicae* J.B.Phipps; not *C.* ser. *Nigrae* (Loudon) Russanov). It differs from black-fruited *C.* ser. *Nigrae* and *C.* sect. *Douglasianae* in thorn diameter, leaf shape, and geographic distribution.

Key to sections in *Crataegus* subg. *Sanguineae*

1. Stipules usually persistent. Inflorescence with falcate bracteoles at the base of lower branches. Mature fruits vary in color from yellow, orange, and red to purple or black. Pyrenes strongly pitted on ventral/radial surfaces. Plants native to Eurasia. .....**sect. *Sanguineae***
— Stipules usually caducous, but sometimes persistent on long shoots. Inflorescence without bracteoles at the base of lower branches. Mature fruits purple, purplish black or black. Pyrenes more or less plane, shallowly pitted or excavated on ventral/radial surfaces. Plants native to North America. 2
2. Thorns slender. Subterminal leaf blades of flowering shoots usually more than 2 times as long as wide. Rocky Mountains and southwestern United States. .....**sect. *Salignae***
— Thorns stout, conic. Subterminal leaf blades of flowering shoots usually less than 1.5 times as long as wide. Pacific Northwest and disjunct in the Upper Great Lakes Basin. .....**sect. *Douglasianae***

Though E. L. Greene (1896) initially noted a probable affinity to *C. rivularis*, *C. saligna* was considered closely related to *C. brachyacantha* by E. J. Palmer (1925) and included in sect. *Brevispinae*, which was accepted by Phipps et al. (1990). Although field observations and a cladistic analysis (of morphological data) led Phipps (1999) to observe that *C. saligna* is allied to *C. rivularis* and *C. erythropoda*, he refrained from concluding that the North American black-fruited *Crataegus* species are monophyletic because of the limited sample of red-fruited out-group species in the analysis. At the same time, however, *C. erythropoda* was the sole and type species of ser. *Cerrones* (Phipps, 1998: 1872) when first published. Subsequently, however, Phipps et al. (2003) included *C. rivularis* in ser. *Cerrones*. Analyses of microsatellite (Dickinson et al., 2008) and a combination of nuclear and plastid loci sequence data (Lo et al., 2009a) led to enlarging ser. *Cerrones* further by adding *C. saligna*, the series thus comprising all three black-fruited species found in the southern Rocky Mountains (Colorado, Idaho, New Mexico, Utah, Wyoming) and adjacent states (Arizona, Nevada; Dickinson et al., 2008). This concept of the series was then used in Flora of North America (Phipps, 2015).

Crataegus sect. *Salignae* includes only one series — ser. *Cerrones* — and forms a clade within *C.* subg. *Sanguineae* sister to members of *C.* sect. *Douglasianae* and *Sanguineae* in phylogenetic analyses of DNA sequence variation in ITS2 (Zarrei et al., 2014), cpDNA loci (Fig. 2; Zarrei et al., 2015; Liston et al., in prep.), and 245 single-copy nuclear loci (Liston et al., in prep.). The section appears to be an agamic complex, in which *C. saligna* is the diploid taxon, and *C. rivularis* and *C. erythropoda* are apomictic allotetraploids whose pollen parents are tetraploid members of red-fruited *C.* subg. *Americanae* (thorns long, calyces abundantly toothed, 10 stamens per flower). The allotetraploids are thus morphologically intermediate in some respects between *C. saligna* and their *C.* subg. *Americanae* parents (Table 2 in Liston et al., in prep.). Nevertheless, all three species demonstrate high morphological affinity (in thorn length and diameter, color of mature twigs and fruits, shape of leaves) that can be easily observed in the field. We do not support the idea of separating *C. rivularis* and *C. erythropoda* from *C. saligna* into nothotaxa of any rank, although we cannot exclude such possibility in future work. Therefore, in order to maintain nomenclatural stability, we chose to describe a new section with an orthospecies *C. saligna* as the type rather than publish a name at new rank.

DISCUSSION

Potter et al. (2007) inferred a North American origin for the Rosaceae as a whole but pointed out the need for detailed studies of the phylogeny and phylogeography of the different tribes of the family. The predominantly Holarctic distributions of large genera in the Maleae (and not just *Crataegus*, Fig. 2) makes it clear that the history of these genera involves one or both of the Bering Land Bridge (BLB) and the North Atlantic Land Bridge (NALB; terminology as in Graham, 2018). Graham uses the large, cosmopolitan non-Rosaceous genus *Ilex* L. as an exemplar of a group for which some data are equivocal, but nevertheless support migration across the BLB and from North America into South America, aided in part by its fleshy red fruits and concomitant bird and mammal dispersal much as is known to occur in

hawthorns (reviewed in Dickinson, 1985). Comparisons can also be made with other fleshy fruited genera like *Toxicodendron* Mill. (Jiang et al., 2019) and *Viburnum* L. (Landis et al., 2020). We envision roles for land bridges for hawthorns, rather than (extreme) long distance dispersal (LDD), because simulations (Nathan, 2006) and observational studies (on *Prunus*; Jordano, 2017) suggest that short to medium distance (up to 10s of km) dispersal events will be much more frequent than ones that are 10 to 100 times longer. Short to medium distance dispersal events also seem more likely to deposit seeds within habitats permitting offspring to germinate and establish. Moreover, given the gametophytic self-incompatibility found in the Maleae diploids (Dickinson et al., 2007), successful spread must have depended on multiple dispersals to any given patch of suitable habitat, in order for newly established individuals to be able to reproduce successfully.

Recent molecular phylogenies of similarly fleshy-fruited *Amelanchier* (Burgess et al., 2015), *Malus* (Nikiforova et al., 2013), and *Sorbus* s. str. (Li et al., 2017) each show sister-group relationships across the BLB. The *Amelanchier* results suggest a North American origin of the genus followed by expansion of two sister clades, one in western North America (clade A; Burgess et al., 2015) and the other, crossing the BLB, into Eurasia (clade O; Burgess et al., 2015). The earliest (Eocene) divergence in *Malus* is between North American *M.* sect. *Chloromeles* (Decne.) Rehder and the rest of the genus, all of which occurs in Eurasia (Nikiforova et al., 2013). Nikiforova et al. also corroborate earlier indications of the uniqueness in North America of *M. fusca* (Raf.) C.K.Schneid. (Dickson et al., 1991; Routson et al., 2012). This Pacific Northwest crabapple, in Asian *M.* sect. *Sorbomalus* Zabel, evidently crossed the BLB from west to east (Nikiforova et al., 2013), probably no earlier than the late Miocene and possibly much more recently (Williams, 1982; Routson et al., 2012). *Sorbus* s. str. diversified in Eurasia, but each of two early diverging clades (*S.* sect. *Sorbus* and *Commixtae* McAll.) contain North American species whose ancestors could have crossed the BLB from west to east as early as the Oligocene or Miocene (Li et al., 2017). Li et al. did not include North American members of *S.* sect. *Tianshanicae* (Kom. ex T.T.Yu) McAll. (*S. occidentalis* (S.Watson) Greene, *S. sitchensis* M.Roem.) in their sample, but if affiliation of these species with *S.* sect. *Tianshanicae* (McAllister, 2005) is confirmed, then this group too is one in which a later crossing of the BLB occurred (late Miocene at earliest; Li et al., 2017).

Crataegus (Fig. 2) appears to resemble its sister genera, *Amelanchier* and Central and South American *Hesperomeles*, in their strong (or exclusive) association with the New World (cf. Evans, 1999). Unlike these other genera, however, the early diversification of *Crataegus* appears to have taken place across the NALB beginning in the Eocene or possibly earlier (Lo et al., 2009a; Lo and Donoghue, 2012; Wen et al., 2016). Ancestors of *C.* subg. *Crataegus* persisted on the east side of the Atlantic but became extinct in North America apart from their modern, apparently hybrid derivatives, *C. marshallii* Eggl., *C. spathulata* Michx., and *C. phaenopyrum* (L.f.) Medik. (Lo et al., 2009a; Phipps, 2015). Extinction of *C.* subg. *Crataegus* in North America is suggested by its absence at present, and the occurrence of fossil leaves resembling those of *C.* subg. *Crataegus* in the late Eocene Florissant Beds of Colorodo (e.g. *C. copeana* MacGinitie; MacGinitie, 1953; iDigPaleo, ongoing). In contrast, the ancestors of *C.* subg. *Brevispinae* persisted on the west side of the Atlantic and became extinct in Eurasia if they were ever present there. *Crataegus* subg. *Americanae* and *Sanguineae*, however, likely arose on the west side of the NALB. Difficulties in resolving which of the earliest arising groups (*C.* subg. *Crataegus*, *Brevispinae*, and *Mespilus*; Fig. 2) is sister to the rest of the genus could be explained by their rapid radiation, with the single species of the latter two subgenera being all that remains from their precursors, on either side of the expanding Atlantic and extinct elsewhere. Long distance dispersal could also explain the presence of the hybrid derivatives of *C.* sect. *Crataegus* in North America but, as noted above, such events seem less likely than either the shorter distance dispersals underlying migrations across land bridges, or a combination of vicariance and extinction events (but we note the evidence for LDD from species with bipolar distributions; Popp et al., 2011; Villaverde et al., 2017). Vicariance related to the disappearance of the NALB and asymmetric extinctions appear to us as better explanations of the geographic relationships of *C.* subg. *Brevispinae* and *Mespilus*. Understanding this history will require

better resolution of the early branching in the phylogeny, and more data from fossils that would provide location and time control.

Fossil wood (*Maloidoxylon* Grambast-Fessard) resembling that of *Amelanchier* and *Crataegus* is known from the Eocene and Miocene of Colorado (Wheeler and Matten, 1977; Wheeler and Manchester, 2002), as well as from the Miocene of Patagonia (Pujana, 2009) and Europe (InsideWood, 2004–onwards; Wheeler, 2011). Fossil leaves attributed to *Crataegus* are known from not only as early as the Eocene of North America (MacGinitie, 1953; Dillhoff et al., 2005; DeVore and Pigg, 2007) but also, from the Oligocene on, in Europe (Paleobiology Database, http://fossilworks.org). Paleogeographic reconstructions in Sanmartin et al. (2001) and Graham (2018) suggest that the NALB was available into the Oligocene so that migration from North America into Eurasia from the west, followed by vicariant diversification on each continent as the North Atlantic widened, seems plausible.

Because leaves are so abundant in the fossil record, it is important for paleobotanists to appreciate the range of leaf morphologies present within just the genus *Crataegus*. The soft x-ray leaf images (Ross, 2008 in Dickinson et al., 2020) in Fig. 3 are a selection from those deposited and freely accessible online (Dickinson et al., 2020). This collection of images augments *Crataegus* leaf images available in the Cleared Leaf Image Database (Das et al., 2014) and elsewhere (e.g. the University of California Museum of Paleontology Cleared Leaf Collection, https://ucmp.berkeley.edu/collections/paleobotany-collection/ucmp-cleared-leaf-collection/), and provides greater detail and more comprehensive taxonomic coverage of the genus than is available elsewhere. It is important to note, however, that the resolution obtained in x-ray images is limited by the size and resolution of the x-ray images. The images are the same size as the leaves themselves, so that resolution is a function here of the grain size of the x-ray film and then, of the resolution of the digital camera that captures the image from the x-ray negative. Digital x-ray imaging is available commercially or can be accomplished using synchrotron (x-) radiation (Blonder et al., 2012). Alternatively, magnified, high resolution digital images of leaf venation can be obtained using the lenses and sensor of a digital camera and chemically cleared and stained leaves (Buechler, 2010; Das et al., 2014; Zhu and Manchester, 2020; Blonder, undated). However, this approach is much more labor intensive, and effectively represents destructive sampling when leaves are obtained from herbarium specimens (cf. Wing, 1992; Dickinson et al., in prep.).

CONCLUSIONS

Whereas there is no debate on *C. brachyacantha* being *Crataegus*, *C. germanica* is often and arguably treated outside of *Crataegus* as the only species of *Mespilus* (Phipps, 2016a, b). Even though one can always find morphological characters to distinguish these two genera, their close relationship is evident from both recent molecular studies (Lo et al., 2007; Zarrei et al., 2015; Liston et al., in prep.) and morphological affinity. Moreover, synapomorphies such as proleptic lateral shoots, presence of thorns, two superposed ovules with only one being fertilized, absence of false locules, and woody endocarp, make them very distinct from the '*Amelanchier*' clade, which appears to be sister to the '*Crataegus*+*Hesperomeles*' clade (Li et al., 2012; Lo and Donoghue, 2012; Liu et al., 2019; Liu et al., 2020). Given the impossibility of finding objective measures of dissimilarity that can be applied universally to discriminate taxonomic ranks and the fact that there is always some arbitrariness in distinguishing such ranks especially at and above the genus level (Stevens, 1997), we believe that accepting *Mespilus* and *Crataegus* as a single genus can only lead to a better understanding of their evolution. A concept of *Crataegus* that embraces *Mespilus* promotes taxonomic stability (Talent et al., 2008; Kurtto et al., 2013) and fosters research programs focused on understanding evolution in all the descendants of a common ancestor.

The current classification of *Crataegus* includes five subgenera corresponding to main lineages discovered by molecular studies (Table 1), each of which, apart from *C.* subg. *Crataegus*, has a largely

stable array of sections and series. Sections in *C.* subg. *Crataegus*, however, are somewhat debatable since no study to date has used sufficient accessions to represent all the putative sections or series in the subgenus. *Crataegus* sect. *Crataegus* sensu K. I. Christensen (1992) seems to be monophyletic and morphologically consistent, whereas *C. pinnatifida* Bunge is most likely sister to it (either included or separated to sect. *Pinnatifidae* Zabel ex C.K.Schneid.). The few molecular data available for *C.* sect. *Cuneatae* Rehder ex C.K.Schneid. and *Hupehenses* J.B.Phipps suggest they should be placed in C. subg. *Crataegus*, but these data fail to suggest what the relationship between *C. cuneata* and *C. hupehensis* Sarg. and *C.* sect. *Crataegus* is. Finally, there are almost no molecular data for *C. scabrifolia* (Franch.) Rehder (*C.* sect. *Henryanae* Sarg.). Those that are available (Du et al., 2019) are suspect because the illustration for their material may suggest inaccurate identification of studied species. Li et al. (2017) included vouchered *C. scabrifolia* as the sole *Crataegus* species among the outgroup taxa in their study of the phylogeny of *Sorbus* s. l., but none of their sequence data are from loci shared with other phylogenetic studies of *Crataegus* to date. Including *C. scabrifolia* in *C.* subg. *Crataegus* has been based up to now entirely on morphological evidence (e.g. occasional presence of leafy thorns, short aphyllous thorns, and more or less persistent stipules on long shoots) and has ignored the unlobed leaves found in this species.

The system we present does not include hybrids between species belonging to different subgenera (or sections). Nothosubgenera have not yet been described. At the sectional level, only *C.* nothosect. *Crataeguineae* K.I.Chr., *Coccitaegus* K.I.Chr. & T.A.Dickinson, *Crataeglasia* K.I.Chr. & T.A.Dickinson, *Phippsara* T.A.Dickinson & E.Y.Y.Lo, and *Crataemespilus* (Camus) T.A.Dickinson & E.Y.Y.Lo are known. Distant hybrids and hybrids with ambiguous parentage in *Crataegus* are to be the main focus of further studies, so some additional sections are very likely to get status of nothosections, and a number of new ones might need to be described. In addition, *C. marshallii*, *C. phaenopyrum*, and *C. spathulata* need to be confirmed as paleohyrbids between some species of *C.* subg. *Americanae* and *Sanguineae* and members of an extinct lineage close to *C.* subg. *Crataegus* (as was suggested by Lo et al., 2009a). Considering that many allotetraploids have yet to be discovered (especially in *C.* subg. *Americanae*), we refrain from providing a comprehensive classification of *Crataegus* with respect to nothotaxa.

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Table 1. Current subgeneric and sectional classification of *Crataegus* (excluding *C. marshallii*, *C. spathulata*, *C. phaenopyrum*, and other intersubgeneric and intersectional hybrids). Includes information for vouchers of hawthorn individuals used here as sources of leaves for x-rays shown in Fig. 3. Ploidy level and other data as per the publications shown. Localities are all in Canada or the U.S.A. Voucher specimens are deposited in the Green Plant Herbarium of the Royal Ontario Museum (TRT). TRT accession numbers are linked to online specimen images (https://crataegus.library.utoronto.ca/***TRTnnnnnnnn.***JPG); M numbers are the online MorphoBank media numbers (http://morphobank.org/permalink/?P1390; http://dx.doi.org/10.7934/P3190). Sections marked with (*) are provisional with very little or no molecular evidence known.

	TRT Accession and MorphoBank numbers (Dickinson et al., 2020)	2*n* (*x*=17); stamen number	Collector and number	Publication	Locality
***Crataegus* L.**					
subg. *Mespilus* (L.) Ufimov and T.A.Dickinson					
sect. *Mespilus* (L.) T.A.Dickinson and E.Y.Y.Lo					
C. germanica (L.) Kuntze	TRT00026644 M584768	2*x* A_{30}	Hess, W., and M. Linden 6220V93	(Evans and Dickinson, 2005; Talent & Dickinson, 2005)	Illinois, DuPage Co. Morton Arboretum (665-80). Cultivated from seed from wild in Tauria, Crimean, State Nikita Bot. Gard., Jalta, Tauria, Ukraine
subg. *Brevispinae* (Beadle) Ufimov and T.A.Dickinson					
sect. *Brevispinae* Beadle ex C.K.Schneid.					
C. brachyacantha Sarg. & Engelm.	TRT00000025 M584760	2–3*x* A_{20}	Reid, C. 5202	(Talent & Dickinson, 2005)	Louisiana, Ouachita Parish. Ouachita WMA, ca. 7.5 miles SE of Monroe
subg. *Crataegus*					
sect. *Crataegus*					
C. laciniata Ucria sensu K.I.Chr.	TRT00002426 M584673	2*x* A_{20}	Dickinson, T. A. s.n.	(Talent & Dickinson, 2005)	Massachusetts, Suffolk Co. Cultivated, Arnold Arboretum (AA238-71A)
C. laevigata (Poir.) DC.	TRT00002174 M584601	2*x* A_{20}	Zika, P. 18472, with A. L. Jacobson and L. Falb	(Talent & Dickinson, 2005)	Washington, San Juan Co. Bird sown in thickets, T36N R2W S19, San Juan Islands, Crane Island, E end
sect. *Pinnatifidae* Zabel ex C.K.Schneid.*					
sect. *Cuneatae* Rehder ex C.K.Schneid.*					
sect. *Hupehenses* J.B.Phipps*					
sect. *Henryanae* (Sarg.) J.B.Phipps*					
subg. *Americanae* El Gazzar					
sect. *Coccineae* Loudon					
C. opaca Hook. & Arn.	TRT00002042 M584679	2*x* A_{20}	Dickinson, T. A. 2003-33, with N. Talent and S. Nguyen	(Talent &Dickinson, 2005; Zarrei et al., 2015)	Louisiana, Sabine Parish. Cultivated

C. triflora Chapm.	TRT00021431 M584762	2*x* A_{30}	Dickinson, T. A. 2003-22, with N. Talent, S. Nguyen and R. Lance	(Talent & Dickinson, 2005)	Alabama, Autauga Co. Jones Bluff, SSW of Peace
sect. *Macracanthae* Loudon					
C. calpodendron (Ehrh.) Medik.	TRT00002039 M584551	2*x* A_{20}	Dickinson, T. A., N. Talent NT166 and E. Garrett	(Talent & Dickinson, 2005)	Ontario, Middlesex Co. Mosa Tp., Conc. Rd. VII-VIII, E of Mosa Side Rd. 8
subg. *Sanguineae* Ufimov **sect. *Salignae* T.A.Dickinson & Ufimov**					
C. saligna Greene	TRT00001047 M584583	2*x* + A_{20}	Dickinson, T. A. 2004-05	(Talent & Dickinson, 2005; Zarrei et al., 2015)	Utah, Duchesne Co. River Road, 4 miles N of Duchesne
sect. *Douglasianae* (Rehder) C.K.Schneid.					
C. suksdorfii (Sarg.) Kruschke	TRT00001805 M584618	2*x* A_{20}	Zika, P. F. 18485	(Talent, 2006; Zarrei et al., 2015)	Washington, Clark Co. ca. 1.5 air miles NNW of Ridgefield
sect. *Sanguineae* Zabel ex C.K.Schneid.					
C. wattiana Hemsl. & Lace	TRT00001881 M584549	4*x* A_{20}	Dickinson, T. A. s.n., and R. C. Evans	(Talent & Dickinson, 2005)	Québec; Cultivated, Jardin Botanique de Montréal, Arboretum (1280-50); det. K.I. Christensen 2011

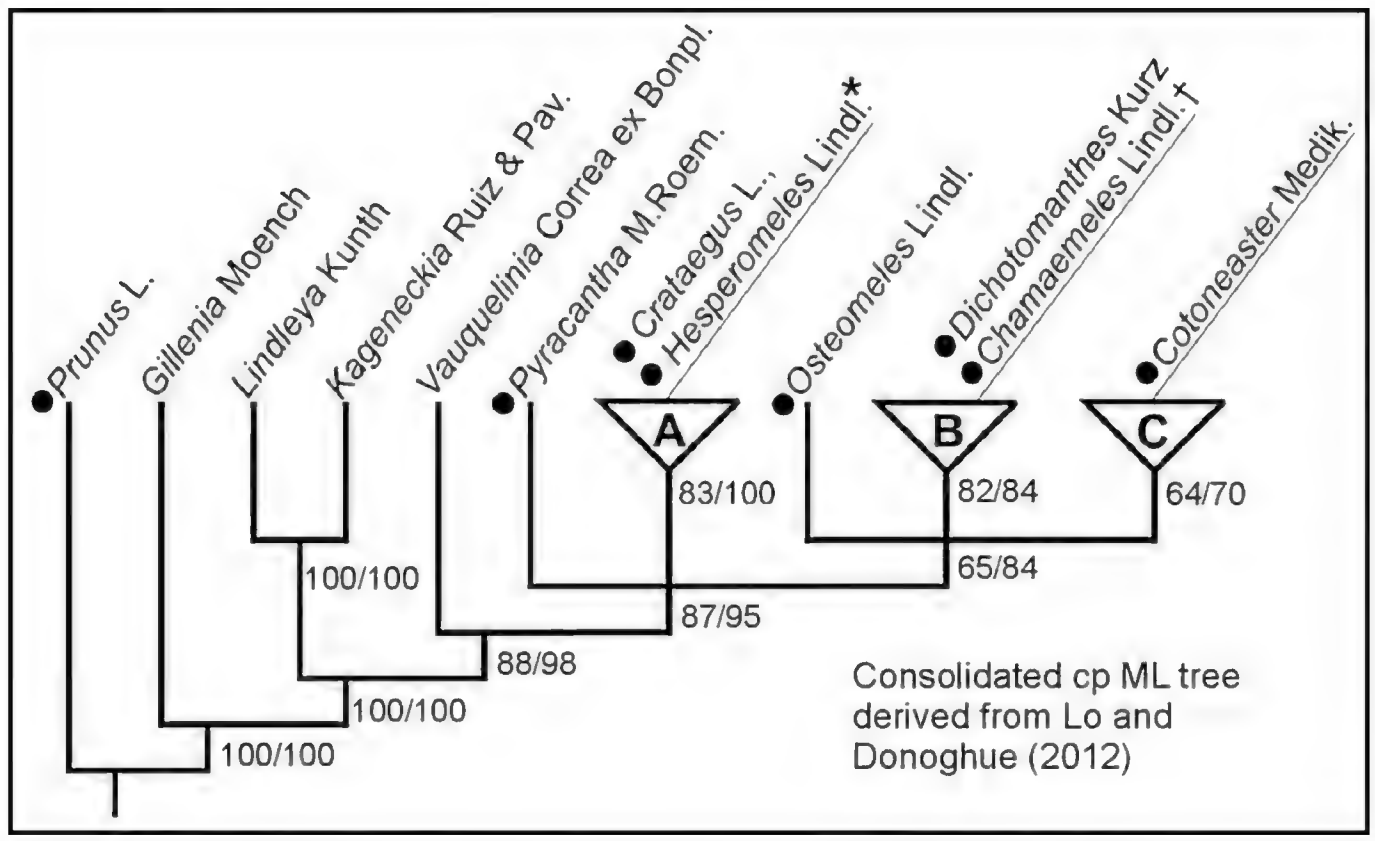
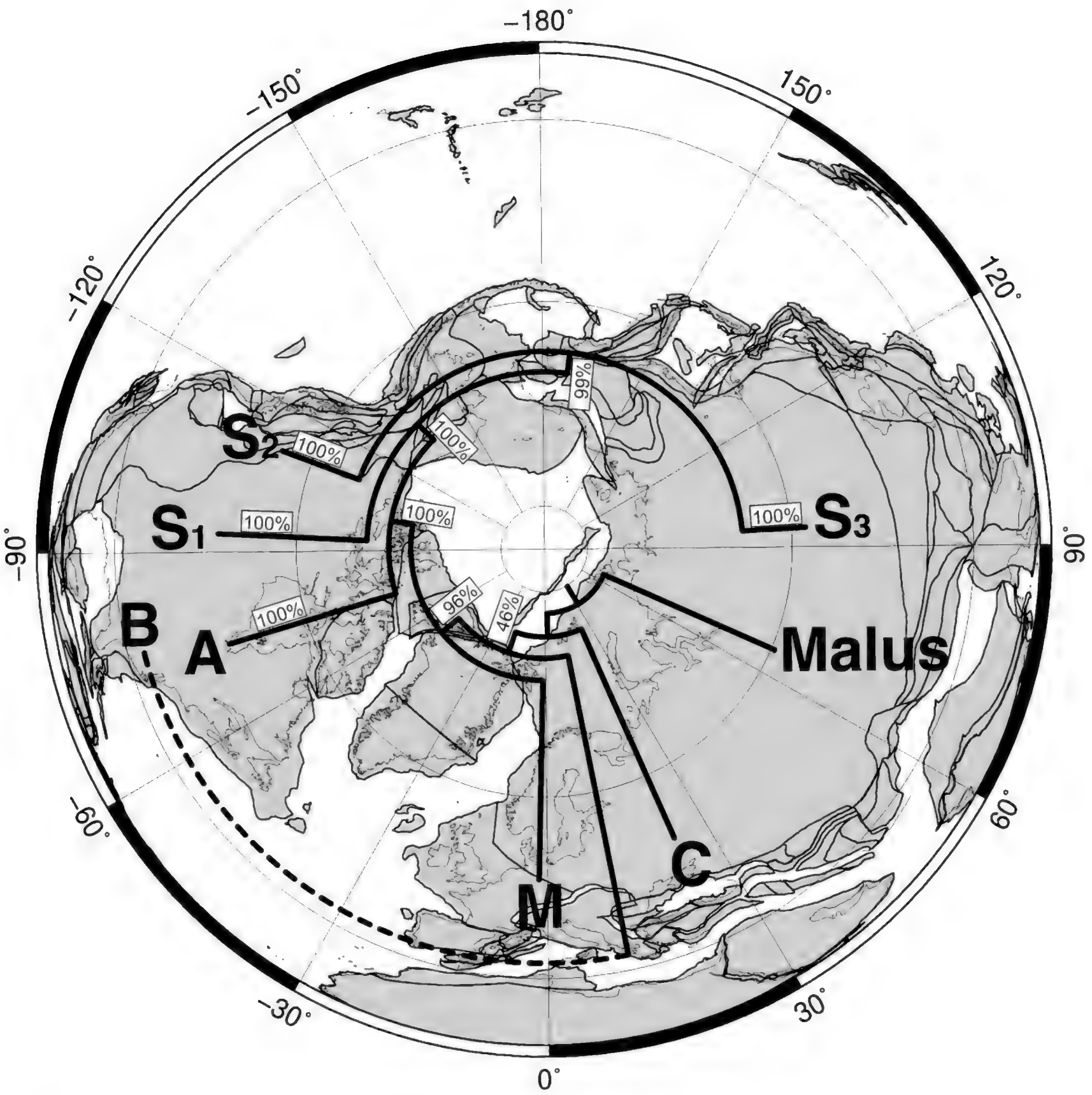
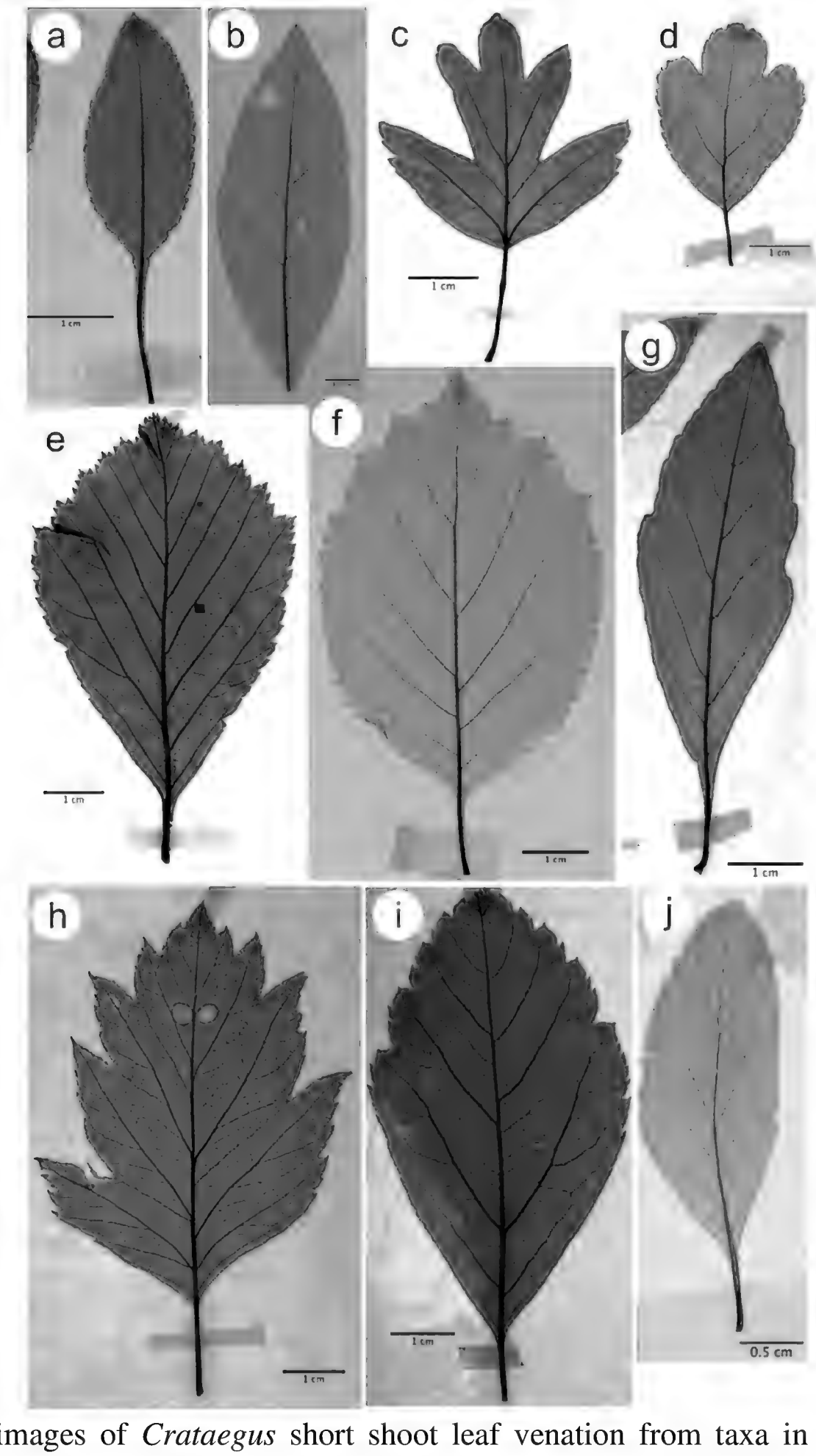


Figure 1. Simplification of the major clades in Rosaceae tribe Maleae (A, B, C; see text for included genera) based on a maximum likelihood tree for 11 plastome loci (coding and non-coding), rooted using species of *Prunus* (left half of figure 1 in Lo and Donoghue, 2012). Branch support indicated by bootstrap (left #) and posterior probability (right #) at nodes. Genera with drupaceous fruits (tribe Crataegeae) indicated by black dots; *Chamaemeles* (dagger) placed on the basis of the results in Li et al. (2012). *Hesperomeles* (asterisk) placed on the basis of the results in Liu et al. (2020).



37 Ma Reconstruction

Figure 2. North polar projection of a tectonic plate reconstruction for 37 Ma produced using the service at www.odsn.de (Hay et al., 1999). Superimposed on the map is the RAxML tree for *Crataegus* subgenera: C, *C.* subg. *Crataegus*; A, *C.* subg. *Americanae*; S, *C.* subg. *Sanguineae*; B (dashed line), *C.* subg. *Brevispinae*; and M, *C.* subg. *Mespilus*. Sections in *C.* subg. *Sanguineae* are labeled S_1, *C.* sect. *Salignae*, S_2, *C.* sect. *Douglasianae*, and S_3, *C.* sect. *Sanguineae*. The RAxML tree was inferred from a complete plastome alignment for a sample of 14 diploid accessions representing all of the infrageneric groups shown here (Table 1; Liston et al., in prep.), rooted using the apple plastome (Velasco et al., 2010), and collapsed as described in the text to show just the subgenera and the three sections within *C.* subg. *Sanguineae*. Support values are > 95% for all nodes except the one supporting *C. brachyacantha* (B; 46%). Labels are placed approximately in the center of the geographic distribution of the corresponding group. Branch lengths are arbitrary.



Figures 3. X-ray images of *Crataegus* short shoot leaf venation from taxa in the infrageneric groups discussed here (Table 1). (a) *Crataegus* subg. *Brevispinae*, *C. brachyacantha* (TRT00000025, M584760); (b) *C.* subg. *Mespilus*, *C. germanica* (TRT00026644, M584768); *Crataegus* subg. *Crataegus*, (c) *C. laciniata* (TRT00002426, M584673); (d) *C. laevigata* (TRT00002174, M584601); *Crataegus* subg. *Americanae*, (e) *C. calpodendron* (TRT00002039, M584551), (f) *C. triflora* (TRT00021431, M584762), (g) *C. opaca* (TRT00002042, M584679); *Crataegus* subg. *Sanguineae*, (h) *C. wattiana* (TRT00001881, M584549). (i) diploid *C. suksdorfii* (TRT00001805, M584618); (j) *C.* sect. *Salignae*, *C. saligna* (TRT00001047, M584583). Scale bars either 0.5 cm (b, j) or 1.0 cm in length (all others). Numbers in parentheses are barcode numbers for specimens in the Green Plant Herbarium (TRT) of the Royal Ontario Museum linked to collection data and online images, and the online MorphoBank media numbers. See Table 1 for taxonomy, details of the voucher specimens and images, and details of the MorphoBank project where x-ray images can be accessed.

Essential oils of trunk, limbs, needles, and seed cones of *Pinus edulis* (Pinaceae) from Utah

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ABSTRACT

Pinus edulis Engelm. (pinyon pine) is an essential oil-bearing evergreen tree. Trunk, limb, needle, and cone samples of pinyon pine from Utah were collected, separately steam distilled, and the resulting essential oils analyzed by GC-FID and GC-MS. The different plant parts shared similar compounds but in widely varying relative percentages. In every sample α-pinene was the most abundant compound, ranging from an average of 50.3% across trunk samples to an average of 70.5% in cone samples. Other prominent compounds included sabinene, β-pinene, δ-3-carene, β-phellandrene, ethyl octanoate, longifolene, and germacrene D, each in varying amounts. Complete profiles and essential oil yields of trunk, limb, needle, and cone samples are established. Published on-line www.phytologia.org *Published on-line www.phytologia.org Phytologia 102(3):200-207 (Sept 21, 2020). ISSN 030319430.*

KEY WORDS: *Pinus edulis,* Pinaceae, pinyon pine, essential oil, Utah, trunk, limbs, needles, cones.

Pinus edulis Engelm., also known as two-needle pinyon pine, is a small evergreen tree in the Pinaceae family (Flora of North America [FNA], 1993). *P. edulis* typically grows at elevations of 1500 – 2700 m and is often shrub-like, though it can grow to 21 m in height, with a wide branching crown; the bark is irregularly furrowed and scaly and there are two blue-green leaves per fascicle (Cronquist et al., 1972; FNA, 1993). It is native to Arizona, Colorado, New Mexico (with smaller populations in California, Wyoming, Oklahoma, and Texas), and Utah, where it often grows alongside *Juniperus osteosperma,* creating the pinyon/juniper forests that are common throughout the region (Cronquist et al., 1972; Ronco, 1990). It is monoecious, except under rare conditions, with most cones forming near the top of the crown (Ronco, 1990). Cones are pale yellow to pale red-brown, 3.5-5 cm, resinous, and mature over 2 years (Cronquist et al., 1972; FNA, 1993). *P. edulis* is a slow growing tree. It reaches full maturity between 75 and 200 years, and the tree can live up to 1000 years and produce seeds for centuries (Ronco, 1990). *P. edulis* has been found to hybridize with *P. monophylla* Torr. & Frém, also known as single-leaf pinyon, where species populations grow in close proximity (Lanner, 1974; Lanner and Phillips III, 1992).

Historically, pinyon pine has been an important tree among many Native American tribes for various reasons. The seeds, commonly called nuts because of their large size of 10-15mm, are an important food source due to their high nutritional value and ease of storage (Bentancourt, 1991; Schellbach, 1933). Schellbach (1933) documented many other practical and medicinal uses of *P. edulis:* the wood has been used for construction, tools, and firewood; the resin of the tree is useful as an adhesive, as well as in making containers waterproof; resin was often used as an antiseptic, protection for cuts and sores, or even to fill cavities; needles were eaten as a purported means to cure syphilis, and ulcers were treated with the powdered gum. Pencil shaped objects made using the gum were also used to extract and treat wounds from projectiles, such as arrows and bullets (Kindscher, 1992). Additionally, pinyon pine had great ceremonial importance in many communities (Schellbach, 1933).

Turpentine distilled from the oleoresin has been previously characterized (Snajberk, 1975; Mirov and Iloff, 1956) and shown to be primarily composed of α-pinene, δ-3-carene, and ethyl octanoate. Analysis of wood monoterpenes also showed high α-pinene, as well as δ-3-carene and limonene (Zavarin

et al., 1989). The needle volatile emission, captured by dynamic headspace, has been characterized as being primarily composed of α-pinene, β-pinene, myrcene, and limonene (Trowbridge et al., 2019). To the authors knowledge, the steam distilled essential oil of the various plant parts has never been fully examined. In this study, the essential oil profiles of *Pinus edulis* seed cones, leaves, limbs, and trunk from Utah are established and compared. Distillation yields of the various plant parts are also reported.

MATERIALS AND METHODS

P. edulis plant material was collected with permission on private land in Tabiona, UT. Three trees in similar states of maturity were chosen and cut 10 cm above ground. Collection details are recorded in Table 1. Voucher samples are held in the Utah Valley University Herbarium (UVSC): *Pinus edulis* Engelm., Wilson 2020-01, -02, -03 (UVSC).

Three whole trees were harvested and separated into cones, needles, limbs, and trunk. Each separated portion was weighed. Cones, defined as the female or seed cone, and needles were distilled whole. Limbs, defined as leafless, 3-5 cm diameter sections nearest the trunk, were cut into segments 2-5 cm in length. The trunk, defined as heartwood, sapwood, cambium, and bark, was chipped. Enough material for three laboratory scale distillations of each plant portion from each tree, for a total of 36 samples from 3 trees (n=3), was retained and stored at -20°C until ready for distillation. All samples were steam distilled.

Laboratory scale distillation was as follows: 3 L of water added to the bottom of a 12 L distillation chamber (Albrigi Luigi S.R.L., Italy), plant material accurately weighed and added to the distillation chamber, distillation for 2 hours by direct steam, and essential oil separated by a cooled condenser and Florentine flask. Essential oil samples were filtered and stored in a sealed amber glass bottle in a cool, dark location until analysis.

Essential oils were analyzed, and volatile compounds identified, by GC/MS using an Agilent 7890B GC/5977B MSD and J&W DB-5, 0.25 mm x 60 m, 0.25 µm film thickness, fused silica capillary column. Operating conditions: 0.1 µL of sample (neat essential oil, 0.1% soln. for C7-C30 alkanes in hexane), 150:1 split ratio, initial oven temperature of 40 °C with an initial hold time of 5 minutes, oven ramp rate of 4.5 °C per minute to 310 °C with a hold time of 5 minutes. The electron ionization energy was 70 eV, scan range 35–650 amu, scan rate 2.4 scans per second, source temperature 230 °C, and quadrupole temperature 150 °C. Volatile compounds were identified using the Adams volatile oil library (Adams, 2007, pdf at www.juniperus.org) using Chemstation library search in conjunction with retention indices. When identifications could not be made with the Adams library, the NIST Mass Spectral Library (version 2.3) was used and KI calculated using C7-C30 alkane standards. Note that limonene/β-phellandrene elute as a single peak, but their amounts are determined by the ratio of masses 68 and 79 (limonene), 77 and 93 (β-phellandrene). Volatile compounds were quantified and are reported as a relative area percent by GC/FID using an Agilent 7890B and J&W DB-5, 0.25 mm x 60 m, 0.25 µm film thickness, fused silica capillary column. Operating conditions: 0.1 µL of sample (50% soln. for essential oils in ethanol, 10% soln. for reference compounds in ethanol, 0.1% soln. for C7-C30 alkanes in hexane), 25:1 split injection, initial oven temperature at 40 °C with an initial hold time of 2 minutes, oven ramp rate of 3.0 °C per minute to 250 °C with a hold time of 3 minutes. For quantification, compounds were identified using retention indices coupled with retention time data of reference compounds.

The percent yield was calculated as the ratio of mass of processed plant material immediately before distillation to the mass of essential oil produced, multiplied by 100.

RESULTS AND DISCUSSION

The aromatic profiles of trunk, limbs, needles, and cones from three *P. edulis* trees are detailed in Table 2. Each reported value is an average from three samples taken from that portion of each individual tree. Essential oil composition is similar in all, but relative percentages differ. Yield is included in Table 3. Cones averaged highest yield (0.7%) and needles averaged lowest (0.01%).

Essential oil obtained by steam distillation of the trunk of three *P. edulis* trees contained, respectively, primarily α-pinene (44.3%, 63.7%, 42.9%), ethyl octanoate (2.9%, 2.5%, 3.3%), and germacrene D (4.9%, 4.4%, 8.1%). Interestingly, the trunk essential oil of tree 1 and tree 3 contained high amounts of longifolene (9.6%, 10.4%) while tree 2 contained only a trace amount. Samples also showed variation in δ-3-carene, with tree 1 containing 10.0%, tree 2 containing 9.0%, and tree 3 containing only 2.9%.

Essential oil obtained from steam distillation of the limbs of three *P. edulis* trees contained, respectively, primarily α-pinene (57.8%, 64.3%, 56.9%), δ-3-carene (9.7%, 6.9%, 3.3%), ethyl octanoate (1.9%, 2.5%, 4.1%), and germacrene D (3.9%, 1.8%, 2.2%). In limb samples as well, longifolene was higher in tree 1 and tree 3 (3.7%, 4.8%), and only trace in tree 2.

Essential oil obtained from steam distillation of the needles of three *P. edulis* trees contained, respectively, primarily α-pinene (56.0%, 62.3%, 52.3%), β-pinene (2.6%, 1.7%, 7.1%), myrcene (3.1%, 1.2%, 1.8%), δ-3-carene (7.3%, 5.4%, 2.7%), beta phellandrene (6.7%, 2.9%, 2.0%), ethyl octanoate (1.7%, 1.7%, 2.7%) and bornyl acetate (0.8%, 3.1%, 4.5%). Consistent with trunk and limb samples, longifolene was lowest in the needles of tree 2.

Essential oil obtained from steam distillation of the cones of three *P. edulis* trees contained, respectively, primarily α-pinene (70.5%, 68.0%, 72.9%), sabinene (3.5%, 2.1%, 1.4%), β-pinene (3.2%, 2.3%, 3.4%), ethyl octanoate (0.9%, 1.3%, 2.6%), and β-bourbonene (0.9%, 1.6%, 2.3%). Longifolene was lowest in tree 2 for the cone samples as well. Cone essential oil of tree 1 and 2 contained high amounts of δ-3-carene (8.4%, 11.0%), while cones from tree 3 contained only 0.6%. It should be noted that the average cone weight for trees 1 and 2 was 6.1g and 8.7g, while average cone weight in tree 3 was 4.7g. Perhaps size of the cones could explain the difference in composition (Table 4).

CONCLUSION

This study confirms that *P. edulis* essential oil is of a similar composition to turpentine (Snajberk, 1975; Mirov and Iloff, 1956), the monoterpene analysis of the wood (Zavarin et al., 1989), and needle volatile emission (Trowbridge et al., 2019), with α-pinene, delta-3-carene, and ethyl octanoate being prominent compounds in the essential oils of all plant parts.

α-pinene is the prominent compound in every portion of *P. edulis,* with the highest amount present in the cones, then limbs, needles, and trunk. Longifolene is highest in the trunk and limbs, though this is not consistent in tree 2. Most cone essential oil samples were high in δ-3-carene, but not in the samples from tree 3. β-phellandrene is most prominent in the needles. Ethyl octanoate is present in similar amounts in essential oils from all sample types. Cones gave the highest yields and needles gave the lowest yields. These results show trends in the essential oil composition of the different parts of *P. edulis* but also that there is some variability despite trees being collected near the same location in similar states of maturity. Further research is needed to determine to cause of this variability.

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Table 1. Collection details for each individual tree.

	***Pinus edulis* #1**	***Pinus edulis* #2**	***Pinus edulis* #3**
date	3/10/2020	4/17/2020	5/1/2020
location	40°19'53" N 110°42'25" W	40°19'53" N 110°42'21" W	40°19'54" N 110°42'15" W
elevation (m)	2036	2036	2033
tree height (cm)	287	432	389
tree width (cm)	183	366	302
trunk weight (g)	13390	21050	20127
cone weight (g)	1420	1345	3036
needle weight (g)	24362	27636	28929
limb weight (g)	31343	29621	21266
total weight (g)	70515	79652	73358

Table 2. Aromatic profile of *P. edulis* essential oil from the trunk, limbs, needles, and cones of three pinyon trees. Each reported value below represents the average of 3 essential oil samples distilled from each portion (trunk, limbs, needles, cones) of the same tree. Compounds detected in one but not all samples are denoted as not detected (nd). Values less than 0.1% are denoted as traces (t). Unidentified compounds less than 0.5% are not included. KI is the Kovat's Index using a linear calculation on DB-5 column (Adams, 2007). Relative area percent is determined by GC-FID.
[1]Identified using the NIST Mass Spectral Library (version 2.3).
[2]Unidentified compound is suspected to be an isomer of ethyl octanoate. The KI was calculated using alkane standards. Prominent peaks in the mass spectrum include: 88 (100%), 101 (61%), 109 (33%), 55 (28%), 73 (25%).

		Pinyon Pine Averages											
		Trunk (%)			**Limbs (%)**			**Needles (%)**			**Cones (%)**		
KI	**Compound:**	1	2	3	1	2	3	1	2	3	1	2	3
921	tricyclene	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.2
924	α-thujene	0.1	t	0.2	0.1	t	0.1	0.1	0.0	0.0	0.1	0.1	0.1
932	α-pinene	44.3	63.7	42.9	57.8	64.3	56.9	56.0	62.3	52.3	70.5	68.0	72.9
945	α-fenchene	t	t	t	t	t	t	0.1	t	t	t	t	t
946	camphene	0.6	0.8	0.5	0.7	0.8	0.7	0.7	0.9	0.9	0.8	0.7	0.8
953	thuja-2,4(10) diene	0.3	0.3	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.3
969	sabinene	0.7	0.1	0.8	1.4	0.2	0.7	1.5	0.5	0.9	3.5	2.1	1.4
974	β-pinene	0.5	0.6	0.5	0.8	0.9	0.8	2.6	1.7	7.1	3.2	2.3	3.4
988	myrcene	0.8	0.9	0.6	1.5	1.0	1.0	3.1	1.2	1.8	0.7	1.4	0.6
1008	δ-3-carene	10.0	9.0	2.9	9.7	6.9	3.3	7.3	5.4	2.7	8.4	11.0	0.6
1014	α-terpinene	0.1	t	0.1	t	nd	nd	t	nd	nd	nd	nd	nd
1020	p-cymene	0.4	0.2	0.3	0.4	0.3	0.5	0.5	0.3	0.2	0.4	0.4	0.3
1024	limonene	2.0	0.6	0.5	2.2	0.6	0.5	3.3	0.5	0.4	3.8	0.7	0.8
1025	β-phellandrene	0.1	0.1	0.1	0.5	0.5	0.2	6.7	2.9	2.0	0.1	0.1	0.1
1026	1,8-cineole	nd	nd	nd	nd	nd	nd	t	t	nd	0.1	t	0.1
1032	(Z)-β-ocimene	0.3	0.5	1.5	0.3	0.2	0.5	0.2	0.2	0.1	0.1	0.1	0.2
1054	γ-terpinene	0.2	0.1	0.2	0.1	t	t	0.1	t	t	nd	nd	nd
[1]1068	methyl 6-methyl heptanoate (NIST 73%)	1.4	0.9	1.4	1.1	1.2	2.2	1.0	1.0	2.2	0.1	0.2	0.2
1086	terpinolene	0.1	t	0.1	0.2	0.3	0.3	0.1	0.3	0.5	0.4	0.4	0.5
1123	methyl octanoate	0.5	0.5	0.6	0.2	0.3	1.1	0.2	0.2	0.7	0.1	0.1	0.2
1122	α-campholenal	0.6	0.5	0.4	0.3	0.4	0.2	0.1	0.2	0.2	0.2	0.3	0.4
1135	trans-pinocarveol	0.6	0.3	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.4	0.6	0.6
[2]1154	unidentified compound	1.2	1.9	0.9	1.3	3.1	2.6	1.7	2.7	2.6	0.2	0.5	0.3
1158	trans-pinocamphone	0.2	0.1	0.1	0.1	0.1	0.0	nd	t	0.1	nd	nd	nd
1166	p-mentha-1,5-diene-8-ol	0.2	0.1	0.3	0.1	0.1	0.1	0.2	0.2	0.1	t	0.1	0.1
1174	terpinene-4-ol	0.1	0.1	0.1	0.2	0.2	0.2	0.4	0.4	0.5	0.1	0.2	0.1
1179	p-cymene-8-ol	0.1	0.1	0.1	t	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
1196	ethyl octanoate	2.9	2.5	3.3	1.9	2.5	4.1	1.7	1.7	2.7	0.9	1.3	2.6
1204	verbenone	0.3	0.2	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.2
1215	trans-carveol	0.1	0.1	0.1	0.1	0.1	t	t	t	t	t	t	0.1

KI	Compound:	Trunk (%)			Limbs (%)			Needles (%)			Cones (%)		
1241	methyl ether carvacrol	nd	t	t	0.1	0.1	t	0.3	0.4	0.4	nd	nd	nd
1287	bornyl acetate	0.7	0.7	0.5	0.4	0.7	0.6	0.8	3.1	4.5	0.3	0.5	0.6
1345	α-cubebene	0.4	0.1	0.5	0.3	0.2	0.4	0.3	0.4	t	0.1	t	0.2
1346	α-terpinyl acetate	0.1	0.1	0.2	0.1	0.1	0.1	0.4	0.2	t	t	0.2	0.1
1374	α-copaene	2.5	0.8	3.5	1.8	0.9	2.5	1.1	0.7	1.6	0.6	0.5	2.0
1380	ethyl-(4E)-decenoate	0.5	t	0.6	0.2	t	0.3	0.1	t	0.3	0.1	nd	0.1
1387	β-bourbonene	t	0.1	t	0.2	0.2	0.1	0.8	0.9	1.4	0.9	1.6	2.3
1389	β-elemene	0.2	0.3	0.3	0.1	0.3	0.3	0.2	0.2	0.2	0.1	0.1	0.2
1395	ethyl decanoate	0.2	0.4	0.5	0.1	0.3	0.4	0.1	0.2	0.3	0.1	0.1	0.2
1407	longifolene	9.6	t	10.4	3.7	t	4.8	1.2	0.1	1.6	0.9	t	2.0
1417	(E)-caryophyllene	0.5	0.5	0.8	0.4	0.3	0.4	0.2	0.3	0.3	0.1	0.2	0.4
1454	E-β-farnesene	0.5	0.5	0.5	0.3	0.4	0.3	0.1	0.2	0.1	nd	0.1	t
1465	(E)-ethyl cinnamate	0.1	0.1	0.2	0.1	nd	0.1	0.1	nd	0.1	nd	nd	0.1
1467	ethyl-(2E, 4Z)-decadienoate	0.4	t	0.7	0.1	t	0.2	0.0	t	0.1	t	t	t
1478	γ-muurolene	0.8	0.5	1.7	0.6	0.7	0.8	0.3	0.7	0.4	0.1	0.2	0.3
1484	germacrene D	4.9	4.4	8.1	3.9	1.8	2.2	0.7	1.2	0.8	0.0	0.2	0.2
1495	γ-amorphene	0.4	0.4	0.5	0.2	0.2	0.2	0.1	0.1	0.1	t	0.1	0.1
1500	α-muurolene	1.0	0.5	1.4	0.7	0.5	0.9	0.4	0.4	0.7	0.2	0.3	0.7
1513	γ-cadinene	0.6	0.4	1.0	0.7	0.9	0.6	0.5	0.9	0.4	0.1	0.1	0.2
1522	δ-cadinene	1.3	0.8	2.7	1.1	0.9	0.8	0.8	0.8	0.3	0.1	0.1	0.3

Table 3. Distribution of mass and essential oil (EO) yield averaged from samples from three *P. edulis* trees. Each tree was cut 10 cm above ground; all measurements and calculations are reflective of above ground portions.

		mass (g)	mass (%)	mass distilled (g)	yield EO (g)	yield EO (%)
Trunk	1	13390	19.0	897.0	3.58	0.4
	2	21050	26.4	1443.5	6.37	0.4
	3	20127	27.4	1295.1	4.93	0.4
	Avg:	18189	24.3	1211.9	4.96	0.4
Limbs	1	31334	44.4	3397.6	5.40	0.2
	2	29621	37.2	3254.9	4.75	0.1
	3	21266	29.0	3810.7	5.35	0.1
	Avg:	27407	36.9	3487.7	5.17	0.1
Needles	1	24362	34.5	1225.1	0.68	0.01
	2	27636	34.7	1434.7	1.24	0.01
	3	28929	39.4	1107.9	0.69	0.01
	Avg:	26976	36.2	1255.9	0.87	0.01
Cones	1	1420	2.0	469.9	3.43	0.7
	2	1345	1.7	445.0	3.01	0.7
	3	3036	4.1	958.7	4.25	0.4
	Avg:	1934	2.6	624.5	3.56	0.6

Table 4. Average weight and number of cones taken from the three pinyon trees.

	Cones		
	Tree 1	Tree 2	Tree 3
weight distilled (g)	469.9	445.0	958.7
# of cones	77	51	202
average cone weight (g)	6.1	8.7	4.7